EXPERT OPINION

informa
healthcare

Osteogenically differentiated mesenchymal stem cells and ceramics for bone tissue engineering

Hajime Ohgushi
Ookuma Hospital, Department of Orthopedics, Hyogo, Japan and Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hyogo, Japan

Introduction: In the human body, cells having self-renewal and multi-differentiation capabilities reside in many tissues and are called adult stem cells. In bone marrow tissue, two types of stem cells are well known: hematopoietic stem cells and mesenchymal stem cells (MSCs). Though the number of MSCs in bone marrow tissue is very low, it can be increased by *in vitro* culture of the marrow, and culture-expanded MSCs are available for various tissue regeneration.

Areas covered: The culture-expanded MSCs can further differentiate into osteogenic cells such as bone forming osteoblasts by culturing the MSCs in an osteogenic medium. This paper discusses osteogenically differentiated MSCs derived from the bone marrow of patients. Importantly, the differentiation can be achieved on ceramic surfaces which demonstrate mineralized bone matrix formation as well as appearance of osteogenic cells. The cell/matrix/ceramic constructs could show immediate *in vivo* bone formation and are available for bone reconstruction surgery.

Expert opinion: Currently, MSCs are clinically available for the regeneration of various tissues due to their high proliferation/differentiation capabilities. However, the capabilities are still limited and thus technologies to improve or recover the inherent capabilities of MSCs are needed.

Keywords: bone marrow, bone regeneration, ceramics, iPS cells, mesenchymal stem cells, regenerative medicine, stem cells, tissue engineering

Expert Opin. Biol. Ther. [Early Online]

1. Introduction

The bone marrow contains certain types of cells which show osteogenic capability. The evidence was obtained by new bone formation resulting from the *in vivo* implantation of the bone marrow [1]. When the marrow was placed on a culture dish, some cells got attached to the dish surface and proliferated to make cell colonies. The cultured cells also showed osteogenic capability. Extensive initial work was done by Friedenstein and his colleagues and they termed the cells as colony-forming units–fibroblastic (CFU-f), which showed extensive proliferation as well as differentiation capabilities including not only osteogenic but also chondrogenic/adipogenic differentiation capabilities [2-4]. In addition, the CFU-f showed myelo-supportive stroma formation. Therefore, the cells are also called marrow stromal cells or mesenchymal stem cells (MSCs) [5-8] and the term 'MSCs' has been used throughout this paper. Due to their osteogenic capability, MSCs are thought to be the ideal cell source for bone tissue engineering. There are many excellent review papers in this field [9-13]. When the MSCs were seeded on various biomaterials and further cultured in an osteogenic culture medium, they differentiated to osteogenic cells which could

10.1517/14712598.2014.866086 © 2013 Informa UK, Ltd. ISSN 1471-2598, e-ISSN 1744-7682

Article highlights.

- Adult stem cells called "Mesenchymal Stem Cells" (MSCs) reside in bone marrow tissue and have capability to differentiate into osteogenic lineage.
- When the MSCs are seeded on various biomaterials and further cultured in an osteogenic culture medium, the MSCs differentiate to osteoblasts which fabricate mineralized tissue on surface of the materials.
- The *in vitro* fabricated mineralized tissue (cultured bone) on materials can show immediate new bone forming capability after their *in vivo* implantation into bony defects and establish tight interface between the materials and pre-existing host bone.
- Due to the *in vivo* new bone forming capability, the cultured bone has been used for various bone reconstruction surgery.

This box summarizes key points contained in the article.

fabricate mineralized tissue on the materials [14-17]. This paper describes our strategies for use of the osteogenically differentiated MSCs derived from patients' bone marrow for their bone reconstruction surgeries [14,18-20].

2. Fabrication of bone tissue using bone marrow MSCs

During mammalian development, bone tissue is formed by enchondral or intramembranous bone formation process [21,22]. The former process is rather complicated due to the necessity for cartilage formation, but the latter process is simple and shows only direct osteogenic differentiation of undifferentiated cells such as MSCs. The differentiation results in the appearance of bone forming osteoblasts, which build the mineralized extracellular matrix (bone matrix). We have developed an experimental method to show *in vivo* bone formation in adult stage. The method utilizes composites of either fresh bone marrow cells [23-26] or the cultured MSCs [27,28] from the marrow and porous hydroxyapatite ceramics (**Figure 1A** and **B**). After *in vivo* implantation of these cells/ceramic composites, either fresh marrow cells or the cultured cells show new bone formation in the pore areas of the ceramics. The process of the bone formation involves: i) appearance of undifferentiated cells on the pore surface of the ceramics; ii) osteogenic differentiation of the cells which results in osteoblasts lining on the ceramic pore surface; iii) mineralized bone matrix formation by the osteoblasts; and iv) mature bone formation in the pore areas [24-26]. Mature bone formation means the existence of bone cells (osteocytes) in small cavities of the bone matrix (lacunae) and many active osteoblasts lining on the matrix surface. During the process of bone formation, cartilaginous tissue could not be detected; thus the process is intramembranous bone formation.

The bone-like tissue could also be fabricated by *in vitro* culture of the MSCs (**Figure 1C**). As reported by Maniatopoulous *et al.* [29], when the primary cultured MSCs were further cultured (sub-culture) in a medium supplemented with beta-glycerophosphate, ascorbic acid and dexamethasone (osteogenic medium), mineralized areas could be detected (**Figure 2**) [30]. After 7 days of the sub-culture, the MSCs showed high alkaline phosphatase (ALP) activity, one of the markers for osteoblast differentiation and clusters of many cuboid-shaped cells were seen. *In situ* hybridization using the osteoblastic maker gene of osteocalcin revealed that these cells were positive for the osteocalcin. The center of the cluster appeared to be an amorphous mineralized area (nodule) [31,32]. After 14 days of culture, these nodules incorporated calcein, which is an indicator of mineralization. Three dimensional analysis of thus formed nodules displayed that the mineralized area directly attached on the culture dish surface and top layer contained many osteoblastic cells and interestingly, the osteocytic cells in small cavities (lacunae) could be detected (**Figure 2**) [32]. The mineralized nodules were also evaluated by physicochemical analyses. X-ray diffraction and Fourier-transform infrared spectroscopy showed that the mineralization was poorly crystalized carbonate containing hydroxyapatite, which exists in normal bone tissue [30]. These findings clearly showed that *in vitro* culture (sub-culture) of the MSCs in the osteogenic medium resulted in bone-like tissue formation and not in simple calcification, although thickness of the *in vitro* mineralized tissue was less than 50 μm and thus not dense. Throughout the culture period, chondrocytes and cartilaginous tissue formation were not detected. Therefore, the *in vitro* process was like intramembranous bone formation as seen in the *in vivo* bone formation by cells/ceramic composites implantation. In this paper, this *in vitro* mineralized tissue by the MSCs is referred to as 'cultured bone'.

3. Rationale for bone tissue reconstruction using the cultured bone

As described in Section 2, both non-cultured freshly isolated bone marrow cells (fresh marrow/ceramic composite) and primary cultured MSCs from the bone marrow (MSCs/ceramic composite) showed *in vivo* bone formation in pore areas of the ceramics, although to achieve abundant bone formation in fresh marrow/ceramic composite, a large amount of fresh marrow cells was needed [23,24]. Incidentally, only a few fresh marrow cells were needed for the culture expansion of MSCs to be used for MSCs/ceramic composites [18,28]. Thus, compared to fresh bone marrow cells, the cultured MSCs are more efficient to show bone formation. The MSCs/ceramic composites are already used for bone reconstruction surgery as first reported by Quarto [33]; however, simple culture-expanded MSCs for clinical application towards bone tissue regeneration [33-35] do not form the main subject of this paper.

Concerning the further culturing (sub-culture) of primary cultured MSCs in the osteogenic medium, which results in

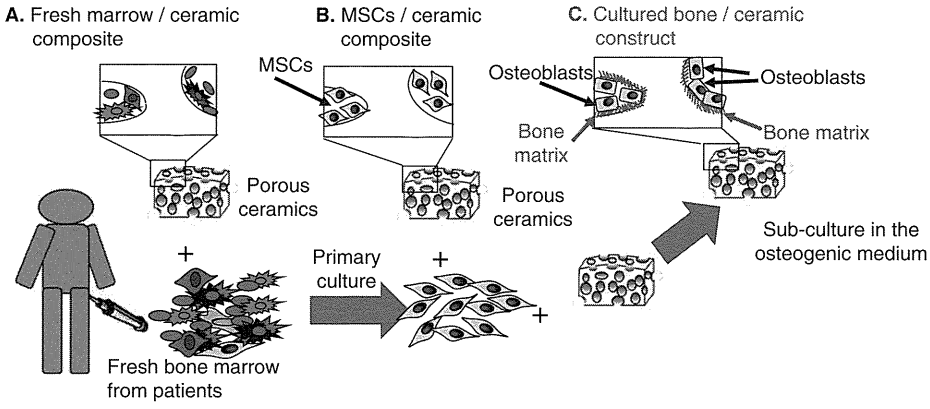

Figure 1. Bone tissue regeneration using patient's bone marrow. Fresh marrow/ceramic (**A**) or MSCs/ceramic composites (**B**) are available for bone tissue regeneration. Number of mesenchymal stem cells (MSCs) from patient's bone marrow can be expanded by culture. When the cultured MSCs are further cultured (sub-culture) in pore areas of the ceramics in an osteogenic medium, the MSCs differentiate into osteoblasts, which fabricate bone matrix on the pore surface (**C**: Cultured bone/ceramic construct). The constructs are also available for clinical applications. Three large rectangular areas are schematic drawings of corresponding small rectangular areas of each composite or construct.

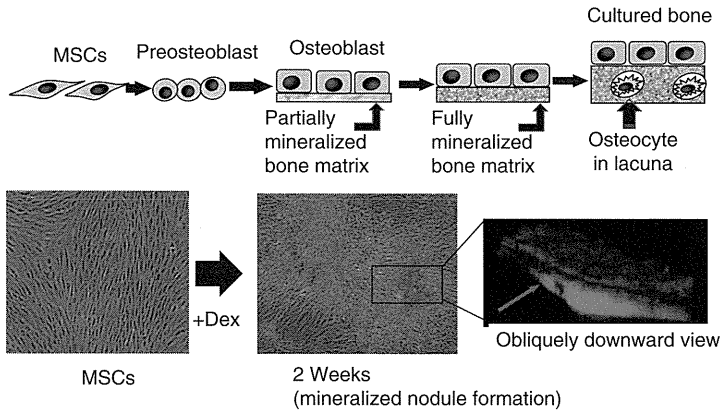

Figure 2. *In vitro* mineralization (cultured bone formation) by culturing MSCs in the osteogenic medium supplemented with dexamethasone (Dex). Upper figures demonstrate schematic cascade of osteogenic differentiation of mesenchymal stem cells in the culture, which finally shows cultured bone formation. Lower figures show phase contrast microscopic views (left two figures) and confocal laser scanning microscopic view (right figure).
These figures are from [32] with permission.

cultured bone formation, it is important to elucidate potential benefits of the cultured bone. The MSCs were sub-cultured in porous ceramics in two different culture media and *in vivo* bone forming capabilities of these ceramics were compared [36,37]. The first medium was a normal one and the other was an osteogenic one supplemented with dexamethasone (Dex). The Dex is key to induce osteogenic differentiation of the MSCs. Therefore, the MSCs cultured in the normal medium without Dex did not show cultured bone formation, whereas the MSCs in the osteogenic medium with Dex showed the *in vitro* cultured bone. As both MSCs were not simply combined with porous ceramics but cultured in the ceramics, the term "construct" was used instead of "composite" for these cultured ceramics (**Figure 1C**). These MSCs/ceramic constructs cultured either in a normal medium or in an osteogenic one were implanted at syngeneic rat subcutaneous sites. One week after implantation of the constructs in the osteogenic medium, they showed histologically obvious thick new bone formation. In contrast, the constructs in the normal medium did not show bone formation. Furthermore, biochemical parameters such as ALP and Osteocalcin were detected much earlier in the constructs in the osteogenic medium compared to those in the normal medium. These results showed that the constructs in the osteogenic medium

had extensive *in vivo* new bone forming capability, because the constructs already contained a prefabricated thin layer of *in vitro* cultured bone on the ceramic pore surface (**Figure 1C**). Thus, the cultured bone/ceramic constructs have immediate *in vivo* osteogenic function [36,37]. A recent paper by Ye *et al.* also confirmed that osteogenically differentiated MSCs showed superior *in vivo* bone forming capability in comparison with non-differentiated MSCs [38].

Another benefit of the cultured bone/ceramic constructs (MSCs cultured on the ceramic pore surface in the osteogenic medium) is that the culture can be done on any kind of porous biomaterial and the cultured bone could be formed in the pore regions of these materials. For example, one of problems of total joint replacement is aseptic loosening of the joints [39,40]. If the loosening progresses, revision surgery is needed and thus morbidity is clear. Especially, non-bioactive ceramics such as alumina ceramics cannot show bone bonding property and sometimes the alumina ceramic total joints exhibit aseptic loosening. In this regard, when the cultured bone was formed on alumina ceramics and implanted into bony defects, tight bone contact between thus formed cultured bone and pre-existing host bone might be established.

To prove this tight bone contact to the alumina ceramic surface, the cultured bone on the porous alumina ceramic surface was fabricated and implanted into rabbit bone defects [41]. The ceramics were alumina ceramic (Kyocera Co., Kyoto, Japan) measuring 15 × 10 × 2 mm. The surfaces were coated with a single layer of alumina beads of 710 – 850 μm diameter. The surface design and composition of alumina ceramics are the same as that used for clinical applications of alumina total ankle prosthesis [42]. After primary culture of the rabbit marrow to obtain MSCs, the MSCs were loaded on the alumina ceramic surface and sub-cultured in the osteogenic medium. The culture process fabricated a thin layer of *in vitro* cultured bone on the alumina surface and after the culture, the alumina ceramics were implanted into the same rabbit tibia defects. Three weeks after the implantation, mechanical bonding and histological examination were performed. Histological examination of the non-cell-loaded implant surfaces showed no bone infiltration into the bone defect. However, the cell-loaded implant surfaces (cultured bone surfaces) exhibited new bone infiltration into the defect. In the mechanical test, the average failure load was 0.60 kilogram-force (kgf) for the non-cell-loaded side and 1.49 kgf for the cultured bone side [41]. These results of the *in vivo* implantation experiments using rabbit bone defect model clearly demonstrated the tight interface between the cultured bone fabricated on porous alumina ceramic surface (cultured bone/ceramic construct) and host bone tissue. Thus, the cultured bone formation on the pore surface of the implants could be used to prevent the loosening of the alumina ceramic total joints. Our recent studies also revealed that the cultured bone could be formed on the pore surface of the metallic implants such as cobalt-chromium based alloy [43]; therefore, the technique can be expanded to solve the problems of various implants in orthopedic applications.

4. Clinical applications of the cultured bone

As described, it has been demonstrated that the cultured bone has extensive osteogenic properties and when it is formed on the pore surface of the alumina/metal implants, it could show tight interface between the surface of the implants and the host bone. Based on these findings, clinical applications of the cultured bone on total alumina ceramic ankle joints were performed [18]. The protocol is as follows: i) Proliferation of MSCs from patient bone marrow by primary culture; ii) sub-culture of the MSCs in the osteogenic medium resulting in the appearance of bone-forming osteoblasts together with bone matrix formation on the alumina ceramic ankle joints (cultured bone formation on the ceramic); and iii) implantation of the tissue engineered ankle joints in the patient (total ankle replacement). The three initial cases using this technology were reported in 2005; since then several such operations have been conducted and patients have shown good clinical scores. The X-ray findings of the representative cases showed that radiodense areas (indicating new bone formation) began to appear around the cultured bone on the alumina ceramics joint ~ 2 – 3 months after the operation. Follow-up X-rays demonstrated that the areas became clearly noticeable (**Figure 3**). The initial radiodense appearance together with a good clinical score proved the establishment of a stable host bone–alumina ceramic interface within a year after the operation. Since then, a durable interface could be achieved [18].

The cultured bone could be formed in pore regions of calcium phosphate ceramics such as hydroxyapatite and beta-tricalcium phosphate ceramics [19,20]. These cultured bones in porous ceramics were used for treatment of bone tumors and osteonecrosis of the femoral head. The osteogenic differentiation of patients' MSCs could be done using various ceramics for clinical applications. The type of the ceramic used was different and dependent on the disease or location of the damaged bone tissues. For example, total joint replacements need ceramics with high mechanical strength but may show loosening; thus, alumina ceramics are used for this purpose. For regeneration of partial bone defects, bioactive calcium phosphate ceramics may be appropriate. Others also used beta-tricalcium phosphate ceramics for alveolar augmentation and hydroxyapatite ceramics for jaw bone defects [44,45].

5. Evidence of *in vivo* osteogenic capability of cultured bone derived from human MSCs

Our basic studies using rat and rabbit MSCs showed extensive new bone forming capability of the cultured bone; however, in considering the clinical applications, evidence of the bone forming capability using human (patient) MSCs is necessary. Under informed consent, bone marrow was aspirated from

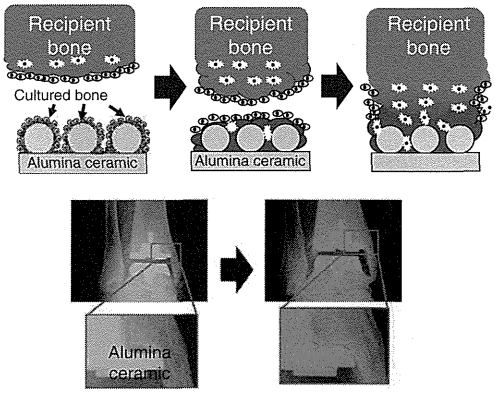

Figure 3. Clinical application of cultured bone on pore surface of alumina ceramic ankle prosthesis. Upper figures show schematic process of bone union between recipient bone and alumina ceramic surface where cultured bone formed. Lower figures show X-ray findings 2 months (left figure) and 1 year (right figure) after implantation.
These figures are from [18] with permission.

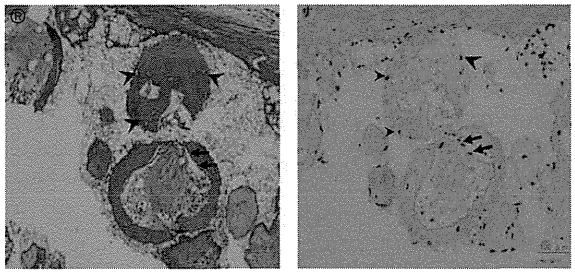

Figure 4. Analyses of the cultured bone/ceramic construct implanted in a nude mouse. Histological section of the construct using hydroxyapatite ceramics was stained by hematoxylin-eosin as seen in the left figure. Osteocytes (arrowheads) appear to be embedded in the newly formed bone matrix and a lining of osteoblasts is seen (arrows). The sections adjacent to the hematoxylin-eosin were used for the *in situ* hybridization seen in the right figure. Brown cells in the sections indicate cells having the human Alu sequence. Arrows: osteoblasts. Arrowheads: osteocytes.
These figures are from [46] with permission.

patients and the number of bone marrow MSCs was expanded by primary culture. The MSCs were further cultured (sub-cultured) in the osteogenic medium in porous calcium phosphate ceramics, which resulted in cultured bone formation on the ceramic pore surfaces (cultured bone/ceramic construct) [46]. Most of the cultured constructs were used for treatment of patients and some of them were implanted into subcutaneous sites of athymic nude mice. The constructs in the mice were harvested after 8 weeks of implantation and used for histological analyses to detect new bone formation. As seen in **Figure 4**, it was examined for human gene of Alu sequence in cells in the pore areas of the ceramics. Many cells in the newly formed bone tissue showed the human signal while some did not, indicating that bone tissue was composed of cells from donor human as well as recipient mouse origin; thus, the bone was hybrid tissue. These findings suggest that cultured bone in the host recipients influenced surrounding undifferentiated mouse cells to differentiate into osteoblasts; therefore, the donor cultured bone survived and fabricated new bone derived from recipient mouse [46].

The survival of human cultured bone after *in vivo* implantation was found by using mouse as recipient. The survival of the cultured bone in a human (patient) recipient was also detected. We treated an 8-month-old girl with rare metabolic bone disease of hypophosphatasia [47]. The hypophosphatasia is caused by mutations of the ALP gene. The mutations are transmitted in an autosomal recessive or dominant manner. The biochemical hallmark of this disease is very low serum ALP activity. The patient showed short limbs together with bowing of the long bones and an abnormally shaped chest. The ALP is a well-known enzyme necessary for bone mineralization, therefore, the patient had low mineralized, fragile bones and showed fractures in many of them.

In one case it was decided to use MSCs from the patient's father for the treatment, because he had normal ALP activity and his MSCs could show *in vitro* as well as *in vivo* osteogenic capability [48]. After the ordinal bone marrow transplantation and administration of immuno-suppressants, the allogenic patient's father's MSCs were intravenously infused. The MSCs were also used for making cultured bone in porous ceramics (cultured bone/ceramic constructs) and were implanted at subcutaneous sites around the knee joints. Seven months after the treatment, the patient needed a second treatment (intravenous infusion) of the MSCs and under the informed consent of the parents, we harvested one of the constructs. A histologic section showed new bone together with osteoblast lining and bone matrix with osteocytes (**Figure 5A**). DNA samples were extracted from the section and analyzed to identify their origin. As the patient was a girl and the donor her father, the detection of male (donor) and female (recipient) DNA signals was attempted. For detecting male genomic DNA by polymerase chain reaction, DYZ1 repeat sequences on the human Y chromosome were used. Each DNA sample from the osteoblast lining and osteocyte areas showed the presence of the DYZ1 sequence (**Figure 5B**). Thus, the osteoblasts and osteocytes were derived from the donor cells that were used for fabrication of the cultured bone [47].

Fluorescence *in situ* hybridization (FISH) was also applied on the phase-contrast image of the section to detect male (XY) and female (XX) signals. As seen in **Figure 5C**, the bone around the ceramic pore surface contained osteocytes of XY signal, whereas the bone away from the pore surface contained XX signal. Thus, two types of bone tissues of donor (male) and recipient (female) origin were seen in the same

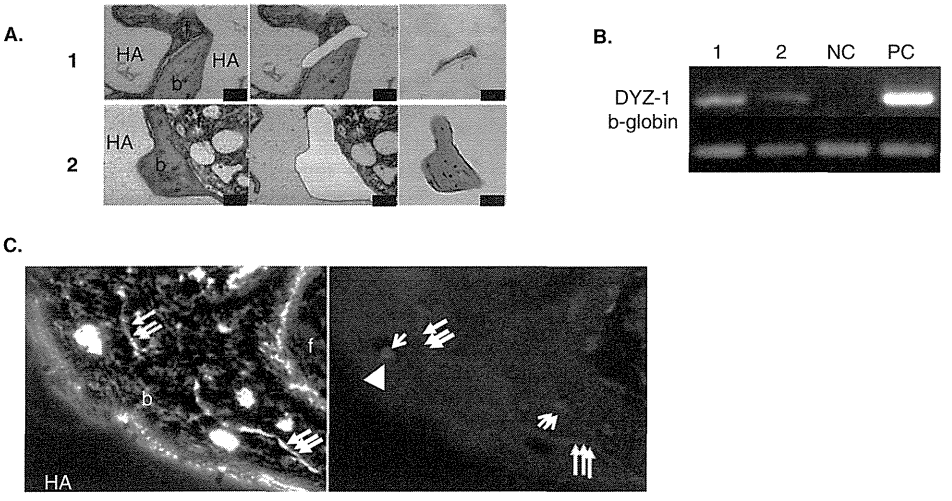

Figure 5. Analyses of the cultured bone/ceramic construct from a patient. A. Areas of the osteoblast lining (1) and osteocytes with bone matrix (2) were dissected from the original section with laser microdissection. The images show the original sections (left), after dissection (middle) and retrieved samples (right). The retrieved samples were used for the detection of the Y chromosome. New bone (b), as well as fibrous tissue (f), can be seen within the pores of the hydroxyapatite ceramic. Bar = 50 μm. **B.** Detection of male-specific DYZ1 on the Y chromosome using dual-PCR analysis. b-globin is an internal control. Lane 1: DNA extracted from the retrieved osteoblast sample as shown in A-1; lane 2: from the retrieved osteocyte sample as shown in A-2; lane 3: negative control (female DNA); lane 4: positive control (male DNA). **C.** Phase contrast (left) and fluorescence (right) images of the retrieved construct on fluorescent *in situ* hybridization. The osteocytes have the donor's (XY) and the patient's (XX) chromosomes. Nuclei are stained with DAPI (blue). The green and red dots indicate the Y chromosome (arrowhead) and the X chromosome (arrows), respectively. New bone (b) and fibrous tissue (f) were observed within the ceramic (HA). Triple arrows indicate the transitional line between donor and recipient derived bone tissues.
These figures are from [47] with permission.

pore areas. There was a transitional interface that marks a line at which the constituents of bone change from donor (XY) to recipient (XX). As there was no intervening fibrous tissue between these two bone tissues, tight interface could be established like appositional bone formation. Therefore, the implanted cultured bone survived/grew and triggered undifferentiated cells of recipient origin to show osteogenic differentiation which started directly on surface of the bone tissue from donor origin [47].

All of these results [46,47] indicate that *in vitro* fabricated cultured bone from human MSCs have *in vivo* osteogenic function to show new bone formation derived from recipient origin, thus the cultured bone/ceramic construct is an osteoinductive material. The osteogenic stimuli might be derived from donor osteoblasts existing on the surface of cultured bone as well as many cytokines including bone morphogenetic proteins in the bone matrix of the cultured bone. Therefore, we speculate that these cellular and protein signals reside in the cultured bone and guide the surrounding recipient tissue to develop into osteogenic tissue and finally show new bone formation from the recipient. Importantly, the bone from the recipient directly faced the bone from the donor as demonstrated by the transitional interface (**Figure 5C**).

The tight interface between donor/recipient bone tissues is clinically important because the cultured bone could also be fabricated in pore regions of non-bioactive alumina ceramics. After the *in vivo* implantation of the cultured bone/alumina ceramic constructs, the bone from the donor could unite to new bone from the recipient. As described earlier, new bone from the recipient was seen even at subcutaneous sites. Therefore, if the cultured bone was implanted into bone defect sites, more new bone from recipient is expected, because abundant MSCs reside in preexisting bone tissue. Thus a stable interface between the ceramics and host bone tissue could be achieved, when implanted into bony defect sites (**Figure 3**). This was clearly demonstrated by the implantation of cultured bone/alumina ceramic constructs into rabbit tibia bone defects as described in Section 3 [41].

6. Osteogenic capability of MSCs from bone marrow and adipose tissue

Mesenchymal stem cells in bone marrow tissue are able to differentiate into various cell types including osteoblasts as described. Recently, MSCs in adipose tissue also have been shown to possess multi-differentiation potential and they are currently used in clinical settings [49-51]. It is reported that

adipose tissue contains more multipotent cells than bone marrow. One gram of adipose tissue yields < 5×10^3 stem cells, which is 100-fold higher than the number of MSCs in bone marrow. Furthermore, adipose MSCs were also used for bone reconstruction surgery [52,53], therefore, it is important to discuss the osteogenic ability of MSCs derived from bone marrow and adipose tissue.

Some previous comparative studies of MSCs from bone marrow and adipose tissue reported no significant difference in osteogenic ability between these MSCs [54] and others reported the inferior osteogenic ability of adipose MSCs when compared to bone marrow MSCs [55]. These previous studies used different experimental protocols. As discussedlater, the differentiation capabilities vary among individuals, therefore, MSCs should be derived from the same donor. However, many utilized different cell sources for the MSCs. To expand the MSC population, cell detachment from the culture dish and re-seeding on another dish is required, and this handling is called passage. The number of passages affects the differentiation ability of the MSCs, however, some used different passage numbers for bone marrow and adipose MSCs to compare the differentiation ability. As more passaged MSCs show lower osteogenic ability, the passage number of the MSCs to be tested should be the same.

The osteogenic ability of bone marrow and adipose MSCs, which were derived from the same donor (Fischer 344 7-week-old male rats) and passaged cells, was studied [56]. To examine the frequency of stem cells in each cell source, the colony-forming units of these MSCs were observed and the number of colonies were counted. It was determined that 1.0×10^6 cells/cm^2 dish for bone marrow MSCs and 1.0×10^4 cells/cm^2 dish for adipose MSCs were the optimal initial cell densities. Therefore, it seems high proliferative cells in adipose tissue are much more than in bone marrow tissue. However, when the primary cultured MSCs were subcultured in an osteogenic medium for 1 and 2 weeks to detect mineralization, ALP activity and osteocalcin contents as osteogenic markers, bone marrow MSCs showed distinct osteogenic differentiation ability in comparison with adipose MSCs. An *in vivo* bone forming assay using the composites of these cells and hydroxyapatite ceramics was also performed. These composites were subcutaneously implanted into syngeneic rats and harvested after 6 weeks. Micro-computed tomographic (CT) and histological analyses demonstrated that new bone formation was easily detected in the composites using bone marrow MSCs, although it was hard to detect in adipose MSCs composites. These results demonstrated the superior osteogenic capability of bone marrow MSCs compared to adipose MSCs [56].

7. MSCs and angiogenesis

As described, MSCs have osteogenic capability in both *in vitro* and *in vivo* conditions. It is well known that osteogenesis and angiogenesis are coupled processes during bone development and new bone appears in close proximity to newly formed vessels [9,57]. It is also reported that inhibition of angiogenesis resulted in impairment of fracture repair [58]. These data indicate that angiogenesis supports osteogenesis. As shown in Section 2, *in vivo* implantation of MSCs/ceramic composites showed new bone formation in the pore areas of the ceramics. The bone formation was seen in the vicinity of newly formed blood vessels. Therefore, *in vivo* osteogenesis of the MSCs also correlated with angiogenesis.

To investigate the origin of new bone in the composites, the MSCs were infected with retroviruses carrying lacZ gene and combined with porous ceramics. The MSCs/ceramic composites were implanted at rat subcutaneous sites [59]. Four weeks after the implantation, the composites were harvested and lacZ expressing cells were stained blue with X-Gal staining. Many osteoblasts and osteocytes around the newly formed bone in the composites were stained blue. Interestingly, endothelial cells in newly formed blood vessels near the bone were also stained blue. Therefore, not only the osteogenic cells but also endothelial cells are derived from donor MSCs [59]. To our knowledge, this is the first report demonstrating coordinate differentiation capability of MSCs into cells of osteogenic as well as endothelial lineage found by ectopic implantation of the MSCs. Evidences of the *in vivo* endothelial differentiation were also demonstrated by implantation of MSCs into damaged heart [60-62] and *in vitro* differentiation of MSCs into endothelial cells were reported as well [63,64].

Angiogenesis requires complex and highly organized interactions between endothelial cells and their environment. Many cytokines including VEGF promote endothelial motility/proliferation to show new vessel formation [9,65]. Fascinatingly, it was reported that VEGF also promoted fracture repair and improved bone formation, and thus could enhance bone regeneration [9,58]. VEGF is produced by many cell types including endothelial cells, fibroblasts, macrophages and so on. Importantly, bone marrow MSCs also produced VEGF more efficiently than bone marrow mononuclear cells [62]; furthermore, human MSCs derived from old-aged donors produced VEGF [66]. The level of the production by human MSCs was about 4 – 5 times more than that by fibroblast and the high production level by human MSCs was maintained during prolonged culture periods (checked up to passage 10) [66]. These data indicate that human MSCs are stable VEGF producing cells.

The osteogenic differentiation capability of the MSCs is clear; in addition, endothelial differentiation and VEGF producing capabilities also reside in the MSCs. Due to these two additional capabilities, rationale for the use of the MSCs in bone regeneration is acceptable. However, current approaches using MSCs or osteogenically differentiated MSCs have limitations to repair large bone defects such as segmental long bone defects, therefore, intensive studies on the role of angiogenesis in promoting osteogenesis are needed.

8. Conclusion

Our basic and clinical experiences showed the usefulness of bone marrow MSCs for bone tissue engineering. Interestingly, MSC culture in the osteogenic medium resulted in *in vitro* cultured bone formation on various material surfaces including ceramics and metallic implants. The cultured bone on alumina ceramic pore surface, for example, could prevent aseptic loosening of the total alumina ceramic ankle joints used in osteoarthritic patients. For these clinical applications, only small amounts of the bone marrow cells were needed for culture expansion of the MSCs. The amount was 10 – 20 ml, which was taken by needle aspiration under local anesthesia. Consequently, a relatively non-invasive technique is required for harvesting the marrow. Other clinical applications using the cultured bone were treatments of bone tumor and osteonecrosis of the femoral head. In these applications, there were no serious adverse effects such as tumor formation and infection around the implantation areas, therefore, bone reconstruction using the cultured bone is a safe and useful approach for patients with bone and joints diseases. These clinical trials were not double-blind, randomized, placebo-controlled studies; thus, extensive further studies are necessary to elucidate the efficacy of our strategy in bone reconstruction surgery.

9. Expert opinion

Our clinical experiences showed the feasibility of patient MSCs to be used for the treatment of osteoarthritis, bone tumor and osteonecrosis. However, some patients' MSCs showed slow proliferation capability. Additionally, osteogenic differentiation capabilities are uneven among patients. Most patients' MSCs showed excellent osteogenic capabilities that resulted in sufficient mineralization after culturing in an osteogenic medium (*in vitro* cultured bone), but others showed minimum capabilities. Thus, individual variation is clear for the proliferation as well as differentiation of patients' MSCs [67].

This variation might be due to agefactors, because our study and those of others showed that decreased bone formation can be observed in old bone marrow cells due to the decreased osteogenic capacity of aged MSCs [68-70]. However, we also experienced high osteogenic capability of MSCs derived from the bone marrow of a patient more than 70 years old [18]. The other factor we should consider is the preparation protocol of the MSCs. We and others isolated/proliferated the MSCs by employing their adhesion property on the culture dish surface. After the culture expansion, the adherent cells were collected. These cells were positive for CD13, CD29, CD44, CD71, CD90, CD105, STRO-1, ALP, HLA class I, SH2, SH3 and SH4, and negative for CD14, CD31, CD34, CD45, and HLA-DR [18,71], and thus they are mesenchymal type cells [72]. Although further purification steps were not utilized in clinical applications, the population of the MSCs was still heterogeneous. In this regard, if there are cell surface markers for genuine MSCs, qualification of MSCs for clinical application is easy and further purification from the culture expanded adherent MSCs could be done.

At present, there are no definitive markers; however, some reported that stage-specific embryonic antigen 1 (SSEA-1) and SSEA-4 are markers for primitive mesenchymal cells [73,74] and also reported the importance of neuronal/lymphoid cell surface CD200 (OX2) glycoprotein expression together with traditional MSC markers [75]. A recent paper showed that the cell surface markers positive to LNGFR, THY-1 and VCAM-1 can be used for the isolation of functional MSCs [76]. Others also reported that stress-tolerant cells reside in dish adherent cells, which can differentiate into cells of all three germ layers. Thus, the cells are multipotent and called multilineage differentiating stress enduring cells (Muse cells), which are positive for SSEA3 and CD105 [77,78]. These unique markers might be available for the purpose of bone tissue engineering in the near future.

Many researchers try to use these cell markers to select more qualified MSCs compared to MSCs currently available. However, there are no well-recognized/established markers for the MSCs and even if the markers are available, it seems the selected MSCs have limited proliferation/differentiation capability, because the MSCs are adult stem cells. In this regard, mostly unlimited proliferation/differentiation capabilities are seen in embryonic stem cells (ES cells) [79]. However, ethical issues are linked to the use of ES cells in basic as well as clinical research [80]. Besides, implantation of the ES cells are allogeneic to patients, and thus the immunological barrier should be considered. It was reported that induced pluripotent stem cells (iPS cells) can be generated from many kinds of cells including human adult cells [81-83]. The proliferation/differentiation capabilities of the iPS cells are comparable to ES cells. The original protocol for iPS cell generation used 3 – 4 gene transduction into adult fibroblasts. The gene transduction may cause chromosomal alteration resulting in the transformation of transduced cells. Recently, protein and chemical compounds have been used for the generation of iPS cells. Therefore, much safer methods compared to the original ones are available for the generation of cells [84-87]. Furthermore, the iPS cells can be generated from the patients, thus the ethical concern is very low and when the patient-derived iPS cells are implanted into the same patient, transplant rejection can be avoided.

Due to the excellent property of the iPS cells, they are expected to be used in many tissue/organ regeneration. In this regard, we reported that MSCs from patients can be frozen stocked for a long period without noticeable loss of viability [88]; we also reported the generation of iPS cells from various frozen stocked MSCs [89-91]. As the MSCs are easily proliferated by culture, the stocked MSCs may be good cell sources for iPS generation when considering their clinical applications. Other points are that MSCs are also generated

from the iPS cells [92-94]; therefore, tissue/organ regeneration might be done using MSCs derived from iPS cells. One problem is that like ES cells, iPS cells can show tumor (teratoma) formation after their *in vivo* implantation, thus it would be safer to implant differentiated cells from the iPS cells rather than undifferentiated iPS cells in order to avoid teratoma formation. As a result, iPS cells at the undifferentiated stage should be eliminated from cell preparation for the purpose of clinical applications. Furthermore, in considering particular clinical applications such as bone tissue regeneration, safe protocols for the osteogenic differentiation from iPS cells are not established. Due to these concerns, there are many problems to be solved for broad applications of the iPS cells in clinical settings.

Well-known iPS cell generation needs transduction of 3 – 4 transcription factors such as Oct3/4, Sox2, Klf4, Nanog and Lin28 into adult cells [81-83]. We reported that single transduction of Nanog or Sox2 gene into human MSCs provoke excellent proliferation as well as osteogenic differentiation capability [95], and thus it could activate the MSC's function. Furthermore, the activated MSCs did not show teratoma after their implantation. This single factor introduction into the MSCs might be a potential tool to solve the problems of MSCs currently available for the purpose of bone tissue engineering in future. More extensive studies concerning the mechanisms of proliferation and differentiation of stem cells are needed to establish safe and effective bone tissue regeneration methods available for patients with various bone and joint diseases.

Acknowledgements

I acknowledge the staff at Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), colleagues at Nara Medical University and doctors at Shimane Medical University. I also thank Professor AI Caplan at Case Western Reserve University. He is the godfather of mesenchymal stem cells.

Declaration of interest

This work was partially supported by Health and Labour Sciences Research Grants, Japan. I have no financial interest to declare in relation to the content of this review paper.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Friedenstein AJ, Shapiro-Piatetzky II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966;16:381-90
2. Friedenstein AJ, Deriglasova UF, Kulagina NN, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2:83-92
3. Friedenstein A. Stromal-hematopoietic interrelationships: maximov's ideas and modern models. Haematol Blood Transfus 1989;32:159-67
4. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 1988;136:42-60
5. Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9(5):641-50
6. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 2005;11(7-8):1198-211
7. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007;213(2):341-7
8. Prockop DJ, Kota DJ, Bazhanov N, Reger RL. Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). J Cell Mol Med 2010;14(9):2190-9
9. Hsiong SX, Mooney DJ. Regeneration of vascularized bone. Periodontology 2000;41:109-22
10. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest 2000;105(12):1663-8
11. Caplan AI. New era of cell-based orthopedic therapies. Tissue Eng Part B Rev 2009;15(2):195-200
12. Amini AR, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. Crit Rev Biomed Eng 2012;40(5):363-408
13. Centeno PJ, Faulkner SJ. The use of mesenchymal stem cells in orthopedics stem cells and cancer. Stem Cells 2012;1:173-9
14. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res 1999;48(6):913-27
• **The paper is review and describes the importance of bone tissue engineering using MSCs and various biomaterials.**
15. Ohgushi H, Dohi Y, Yoshikawa T, et al. Osteogenic differentiation of cultured marrow stromal stem cells on the surface of bioactive glass ceramics. J Biomed Mater Res 1996;32(3):341-8
16. Kotobuki N, Ioku K, Kawagoe D, et al. Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics. Biomaterials 2005;26(7):779-85
17. Kotobuki N, Kawagoe D, Nomura D, et al. Observation and quantitative analysis of rat bone marrow stromal cells cultured in vitro on newly formed transparent beta-tricalcium phosphate. J Mater Sci Mater Med 2006;17(1):33-4
18. Ohgushi H, Kotobuki N, Funaoka H, et al. Tissue engineered ceramic artificial joint–ex vivo osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. Biomaterials 2005;26(22):4654-61
•• **The paper describes the first clinical trials using cultured bone. The patient's MSCs were cultured on alumina ceramic prosthesis and used for treatments of osteoarthritic ankle joints.**
19. Morishita T, Honoki K, Ohgushi H, et al. Tissue engineering approach to the treatment of bone tumors: three cases of cultured bone grafts derived from

patients' mesenchymal stem cells. Artif Organs 2006;30(2):115-18

20. Kawate K, Yajima H, Ohgushi H, et al. Tissue-engineered approach for the treatment of steroid-induced *osteonecrosis of the femoral head:* transplantation of autologous mesenchymal stem cells cultured with beta-tricalcium phosphate ceramics and free vascularized fibula. Artif Organs 2006;30(12):960-2
21. Mackie EJ, Ahmed YA, Tatarczuch L, et al. Endochondral ossification: how cartilage is converted into bone in the developing skeleton Int J Biochem Cell Biol. 2008;40(1):46-62
22. Bruder SP, Caplan AI. Cellular and molecular events during embryonic bone development. Connect Tissue Res 1989;20(1-4):65-71
23. Ohgushi H, Goldberg VM, Caplan AI. Heterotopic osteogenesis in porous ceramics induced by marrow cells. J Orthop Res 1989;7(4):568-78
24. Ohgushi H, Okumura M, Tamai S, et al. Marrow cell induced osteogenesis in porous hydroxyapatite and tricalcium phosphate: a comparative histomorphometric study of ectopic bone formation. J Biomed Mater Res 1990;24(12):1563-70
25. Okumura M, Ohgushi H, Tamai S. Bonding osteogenesis in coralline hydroxyapatite combined with bone marrow cells. Biomaterials 1991;12(4):411-16
26. Okumura M, Ohgushi H, Dohi Y, et al. Osteoblastic phenotype expression on the surface of hydroxyapatite ceramics. J Biomed Mater Res 1997;37(1):122-9
27. Ohgushi H, Okumura M. Osteogenic capacity of rat and human marrow cells in porous ceramics. Experiments in athymic (nude) mice. Acta Orthop Scand 1990;61(5):431-4
28. Kotobuki N, Katsube Y, Katou Y, et al. In vivo survival and osteogenic differentiation of allogeneic rat bone marrow mesenchymal stem cells (MSCs). Cell Transplant 2008;17(6):705-12
29. Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res 1988;254(2):317-30
30. Ohgushi H, Dohi Y, Katuda T, et al. In vitro bone formation by rat marrow cell culture. J Biomed Mater Res 1996;32(3):333-40
31. Uchimura E, Machida H, Kotobuki N, et al. In-situ visualization and quantification of mineralization of *cultured osteogenetic cells.* Calcif Tissue Int 2003;73(6):575-83
32. Kihara T, Oshima A, Hirose M, et al. Three-dimensional visualization analysis of in vitro cultured bone fabricated by rat marrow mesenchymal stem cells. Biochem Biophys Res Commun 2004;316(3):943-8
33. Quarto R, Mastrogiacomo M, Cancedda R, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385-6
34. Marcacci M, Kon E, Moukhachev V, et al. Stem cells associated with macroporous bioceramics for long bone repair: 6- To 7-year outcome of a pilot clinical study. Tissue Eng 2007;13:947-55
35. Shayesteh YS, Khojasteh A, Soleimani M, et al. Sinus augmentation using human mesenchymal stem cells loaded into a beta-tricalcium phosphate/hydroxyapatite scaffold. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106(2):203-9
36. Yoshikawa T, Ohgushi H, Tamai S. Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. J Biomed Mater Res 1996;32(3):481-92
37. Yoshikawa T, Ohgushi H, Nakajima H, et al. In vivo osteogenic durability of cultured bone in porous ceramics: a novel method for autogenous bone graft substitution. Transplantation 2000;69(1):128-34
38. Ye X, Yin X, Yang D, et al. Ectopic bone regeneration by human bone marrow mononucleated cells, undifferentiated and osteogenically differentiated bone marrow mesenchymal stem cells in beta-tricalcium phosphate scaffolds. Tissue Eng Part C Methods 2012;18(7):545-56
39. Rhee YG, Cho NS, Parke CS. Impaction grafting in revision total elbow arthroplasty due to aseptic loosening and bone loss. J Bone Joint Surg Am 2013;95(11):741-7
40. Hasegawa M, Ohashi T, Tani T. Poor outcome of 44 cemented total hip arthroplasties with alumina ceramic heads: clinical evaluation and retrieval analysis after 10-16 years. Acta Orthop Scand 2001;72(5):449-56
41. Tohma Y, Tanaka Y, Ohgushi H, et al. Early bone in-growth ability of alumina *ceramic implants loaded with tissue-*engineered bone. J Orthop Res 2006;24(4):595-603
42. Takakura Y, Tanaka Y, Kumai T, et al. Ankle arthroplasty using three generations of metal and ceramic prostheses. Clin Orthop Relat Res 2004;424:130-6
43. Ogawa M, Tohma Y, Ohgushi H, et al. Early fixation of cobalt-chromium based alloy surgical implants to bone using a tissue-engineering approach. Int J Mol Sci 2012;13(5):5528-41
44. Ueda M, Yamada Y, Ozawa R, et al. Clinical case reports of injectable tissue-engineered bone for alveolar augmentation with simultaneous implant placement. Int J Periodontics Restorative Dent 2005;25(2):129-37
45. Meijer GJ, de Bruijn JD, Koole R, et al. Cell based bone tissue engineering in jaw defects. Biomaterials 2008;29(21):3053-61
46. Matsushima A, Kotobuki N, Tadokoro M, et al. In vivo osteogenic capability of human mesenchymal cells cultured on hydroxyapatite and on beta-tricalcium phosphate. Artif Organs 2009;33(6):474-81
47. Tadokoro M, Kanai R, Taketani T, et al. New bone formation by allogeneic mesenchymal stem cell transplantation in a patient with perinatal hypophosphatasia. J Pediatr 2009;154(6):924-30
48. Katsube Y, Kotobuki N, Tadokoro M, et al. Restoration of cellular function of mesenchymal stem cells from a hypophosphatasia patient. Gene Ther 2010;17(4):494-502
49. Gimble JM, Katz AJ, Bunnell BA, et al. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249-60
50. Gir P, Oni G, Brown SA, et al. Human adipose stem cells: current clinical applications. Plast Reconstr Surg 2012;129:1277
51. Mizuno H, Tobita M, Uysal AC. Concise review: adipose-derived stem cells as a novel tool for future

regenerative medicine. Stem Cells 2012;30(5):804-10

52. Mesimäki K, Lindroos B, Törnwall J, et al. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. Int J Oral Maxillofac Surg 2009;38:201-9

53. Sándor GK. Tissue engineering of bone: clinical observations with adipose-derived stem cells, resorbable scaffolds, and growth factors. Ann Maxillofac Surg 2012;2(1):8-11

54. De Ugarte DA, Morizono K, Elabarbary A, et al. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 2003;174:101-9

55. Gun-II I, Shin YW, Lee KB. Do adipose tissue–derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow–derived cells? Osteoarthritis Cartilage 2005;13:845-53

56. Hayashi O, Katsube Y, Hirose M, et al. Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. Calcif Tissue Int 2008;82(3):238-47

57. Caplan AI. Cartilage begets bone versus endochondral myelopoiesis. Clin Orthop Relat Res 1990;261:257-67

58. Street J, Bao M, deGuzman L, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci USA 2002;99:9656-61

59. Akahane M, Ohgushi H, Kuriyama S, et al. Hydroxyapatite ceramics as a carrier of gene-transduced bone marrow cells. J Orthop Sci 2002;7(6):677-82

60. Nagaya N, Fujii T, Iwase T, et al. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004;287(6):H2670-6

61. Silva GV, Litovsky S and Assad JA, et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 2005;111:150-6

62. Nagaya N, Kangawa K, Itoh T, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112(8):1128-35

63. Oswald J, Boxberger S, Jørgensen B, et al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 2004;22(3):377-84

64. Janeczek Portalska K, Leferink A, Groen N, et al. Endothelial differentiation of mesenchymal stromal cells. PLoS One 2012;7(10):e46842

65. Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. Crit Rev Oncol Hematol 2007;62:179-213

66. Kagiwada H, Yashiki T, Ohshima A, et al. Human mesenchymal stem cells as a stable source of VEGF-producing cells. J Tissue Eng Regen Med 2008;2(4):184-9

67. Ohgushi H, Kitamura S, Kotobuki N, et al. Clinical application of marrow mesenchymal stem cells for hard tissue repair. Yonsei Med J 2004;45(Suppl): 61-7

68. Inoue K, Ohgushi H, Yoshikawa T, et al. The effect of aging on bone formation in porous hydroxyapatite: biochemical and histological analysis. J Bone Miner Res 1997;12(6):989-94

69. Kim M, Kim C, Choi YS, et al. Age-related alterations in mesenchymal stem cells related to shift in differentiation from osteogenic to adipogenic potential: implication to age-associated bone diseases and defects. Mech Ageing Dev 2012;133(5):215-25

70. Bajek A, Czerwinski M, Olkowska J, et al. Does aging of mesenchymal stem cells limit their potential application in clinical practice? Aging Clin Exp Res 2012;24(5):404-11

71. Kotobuki N, Katsube Y, Hirose M, et al. Surface marker expression of human. IFMBE Proc 2005;10:118-20

72. da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells 2008;26(9):2287-99

73. Anjos-Afonso F, Bonnet D. Nonhematopoietic/endothelial SSEA-1 cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. Blood 2007;109:1298-306

74. Gang EJ, Bosnakovski D, Figueiredo CA, et al. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007;109:1743-51

75. Wright GJ, Jones M, Puklavec MJ, et al. The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. Immunology 2001;102:173-9

76. Mabuchi Y, Morikawa S, Harada S, et al. LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. Stem Cell Rep 2013;1(2):152-65

77. Kuroda Y, Kitada M, Wakao S, et al. Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci USA 2010;107(19):8639-43

78. Kuroda Y, Wakao S, Kitada M, et al. Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. Nat Protoc 2013;8(7):1391-415

79. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-7

80. Cohen CB. Ethical and policy issues surrounding the donation of cryopreserved and fresh embryos for human embryonic stem cell research. Stem Cell Rev 2009;5(2):116-22

81. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-76

82. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-72

83. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-20

84. Rhee YH, Ko JY, Chang MY, et al. Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. J Clin Invest 2011;121(6):2326-35

85. Ban H, Nishishita N, Fusaki N, et al. Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. Proc Natl Acad Sci USA 2011;108(34):14234-9

86. Mandal PK, Rossi DJ. Reprogramming human fibroblasts to pluripotency using

modified mRNA. Nat Protoc 2013;8(3):568-82

87. Hou P, Li Y, Zhang X, et al. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 2013;341(6146):651-4

88. Kotobuki N, Hirose M, Machida H, et al. Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng 2005;11(5-6):663-73

89. Aoki T, Ohnishi H, Oda Y, et al. Generation of induced pluripotent stem cells from human adipose-derived stem cells without c-MYC. Tissue Eng Part A 2010;16(7):2197-206

90. Oda Y, Yoshimura Y, Ohnishi H, et al. Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. J Biol Chem 2010;285(38):29270-8

91. Ohnishi H, Oda Y, Aoki T, et al. A comparative study of induced pluripotent stem cells generated from frozen, stocked bone marrow- and adipose tissue-derived mesenchymal stem cells. J Tissue Eng Regen Med 2012;6(4):261-71

92. Lian Q, Zhang Y, Zhang J, et al. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. Circulation 2010;121(9):1113-23

93. Tran NT, Trinh QM, Lee GM, et al. Efficient differentiation of human pluripotent stem cells into mesenchymal stem cells by modulating intracellular signaling pathways in a feeder/serum-free system. Stem Cells Dev 2012;21(7):1165-75

94. Hynes K, Menicanin D, Han J, et al. Mesenchymal stem cells from iPS cells facilitate periodontal regeneration. J Dent Res 2013;92(9):833-9

95. Go MJ, Takenaka C, Ohgushi H. Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities. Exp Cell Res 2008;314(5):1147-54

Affiliation

Hajime Ohgushi[1,2] MD
[1]Department Head,
Ookuma Hospital,
Department of Orthopedics,
2-17-13 Kuise-honmachi, Amagasaki City,
Hyogo 660-0814, Japan
Tel: +81-6-6481-1667;
Fax: +81-6-6481-4234;
[2]Invited Senior Research Scientist,
Health Research Institute,
National Institute of Advanced Industrial Science and Technology (AIST), 3-11-46 Nakouji,
Amagasaki City, Hyogo 661-0974, Japan
Tel: +81-6-6494-7807;
Fax: +81-6-6491-5028;
E-mail: hajime-ohgushi@aist.go.jp