Review Article

Bone tissue engineering using patient's mesenchymal cells: From cellular engineering to gene manipulation

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Mesenchymal stromal cells (MSCs) derived from human bone marrow have capability to differentiate into cells of mesenchymal lineage. Especially, the differentiation capability towards osteogenic/chondrogenic cells is very well known. We have already used the patient's MSCs for the treatments of various patients who have osteoarthritis, bone necrosis and bone tumor. However, the proliferation and differentiation capability of the MSCs are variable and many cells lose their capabilities after several passages. With the aim of conferring higher capability on human bone marrow MSCs, we introduced the Sox2 gene into the cells and found that Sox2-expressing MSCs showed consistent proliferation and osteogenic capability in culture media containing basic fibroblast growth factor (bFGF) compared to control cells. We also found that Nanog-expressing cells even in the absence of bFGF had much higher capabilities for expansion and osteogenesis than control cells. Present paper describes our bone tissue engineering strategy and focuses on the importance of transcription factors for the function of MSCs.

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Introduction

Bone is formed by cells called osteoblasts which arise from progenitor cells in a multistep lineage cascade. At bone defect sites, the osteoblast progenitors are recruited from tissues of outside the bone and inside the bone (periosteum and bone marrow, respectively) and they become osteoblasts through a series of controlled differentiation steps[1,2]. These progenitor cells are, herein, referred to as mesenchymal stromal cells (MSCs) and have capability to differentiate into mesenchymal phenotypes, including osteogenic and chondrogenic cell types. The MSCs can be expanded by tissue culture technique with small amount of human fresh bone marrow cells[3,4]. The culture expanded MSCs have capability to differentiate into active osteoblasts, which fabricate *in vitro* bone matrix consisting of hydroxyaptatite crys-

tals[5-7]. The osteoblasts/bone matrix formation (cultured bone) can show continuous new bone formation after *in vivo* implantation[8,9]. Importantly, the *in vitro* differentiation can be achieved by culturing on various materials including bioinert alumina ceramics[10].

Recent reports also evidenced that the MSCs can show differentiation capability into hepatocyte, neural cells and mores[11-14]. Thus, MSCs can also be called as mesenchymal stem cells[15]. The present paper reviews the MSCs' proliferation and differentiation especially osteogeniec differentiation capability of human MSCs in view points of our clinical experiences. Although, the MSCs have high proliferation and differentiation capabilities, the cells are not genuine stem cells, because after several passages, the MSCs show very slow proliferation and hardly show differentiation into specific cell types such as osteocytes/chondrocyte. Thus, these capabilities of MSCs are limited and it is also discussed in this paper.

Bone tissue engineering using MSCs

Autologous bone grafts are considered as the gold standard for use in treating bone defects, however morbidity is an issue in harvesting healthy tissue (Table 1). The use of allogeneic bone grafts is an alternative method but transplantation immunity as well as the possibility of transmitted diseases cannot be ignored. Recently, synthetic biomaterials such as calcium phosphate ceramics have been used as artificial bone graft materials. Although these materials are known to be biocompatible, they usually do not have the capability of forming new bone[3]. To overcome these problems, tissue engineering is gaining interest as it is applied for regeneration of bone tissue[9]. Tissue engineering utilizes living cells as engineering materials, for example, osteogenic cells such as mesenchymal stromal cells (MSCs) can be combined with artificial bone graft materials[3,4], which can show new bone forming capability (Fig.1).

Table 1 Bone grafts

Conventional methods
A:Autologous bone transplantation
- **Potential for bone regeneration**
- **Easy to model**
- **Bone harvesting damages healthy body parts**
- **Limit to transplantable quantity**

B:Allotransplant of bone
- **Large quantities available, easy to model**
- **Nonuniformity of products (sex, age)**
- **Issues of antigenicity and infection**

C:Artificial bone graft materials
- **Uniform in quality**
- **No limit to amount of use**
- **No capability of new bone formation**

Tissue engineering approaches
D: Bone graft materials + autologous MSC
- **No rejection**
- **Capability of new bone formation**

E:Regenerative cultured bone
- **No rejection**
- **Fabrication of bone matrix + active osteoblasts**
- **Capability of prompt new bone formation**

We advanced the tissue engineering approach and established the *ex vivo* bone tissue construction on the various biomaterials (regenerative cultured bone), which posses *in vivo* new bone forming capability (Fig.2)[8,9]. As shown in the figure, when cultured MSCs were seeded onto the surface of bioactive materials such as hydoroxyapatite ceramics and further cultured in the osteogenic meicium described later, the MSCs differentiated into osteoblasts, which secreted bone matrix containing hydroxyaptatite (HA) crystal on the ceramics[7]. The osteoblasts/bone matrix on the ceramics is referred as cultured bone. When implanted *in vivo*, this cultured bone continued to fabricate new bone[8,9]. Likewise, such *in vitro* differentiated cells on non-bioactive materials such as alumina ceramics provide such ceramics with a covering of the *in vitro* cultured bone[10]. On both bioactive and non-bioactive materials, HA crystals, biologic factors, and osteoblasts are all produced and derived from the donor cells. Thus, *in vitro* cultured bone on the surface of ceramics functions with bone bonding together with new bone-forming capability. The bonding capability is derived from HA crystals in the bone matrix, and the new bone-forming capability is attributed to osteoblasts and many biologic factors, including bone morphogenetic proteins (BMPs). Using this technique, we can alter the surface of non-bioactive materials through bioactive substances having osteogenic function.

These evidences using tissue engineering approach encouraged us for the use of *in vitro* formed cultured bone in clinical applications.

Our clinical experiences

Our approach utilized patient's MSCs and consists of three steps: 1) Proliferation of mesenchymal cells from the patient's bone marrow by culture; 2) Osteogenic differentiation of the culture expanded cells resulting in the appearance of bone-forming osteoblasts together with bone matrix formation on the various ceramics (cultured bone) and 3) Implantation of the cultured bone in the same patients[16]. To obtain the MSCs, we aspirated the patient's bone marrow by needle and cultured it in a culture

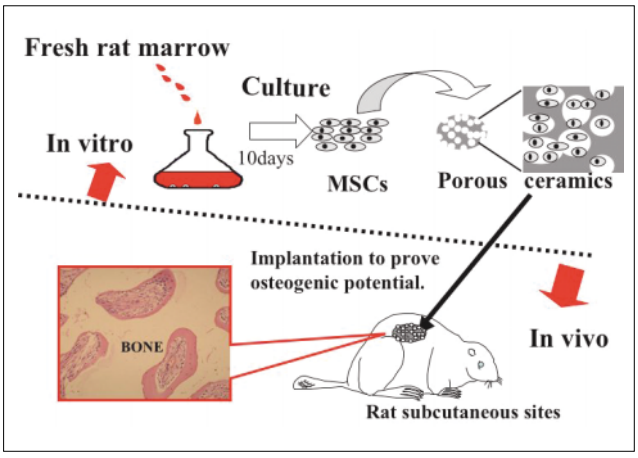

Fig.1 Fabrication of osteogenic materials using mesenchymal stromal stem cells (MSCs)

After culture expansion of marrow MSCs, the cells were combined with porous ceramics, and then implanted at rat subcutaneous sites. After about 4 weeks, new bone formation was seen inside the pore areas of the ceramics.

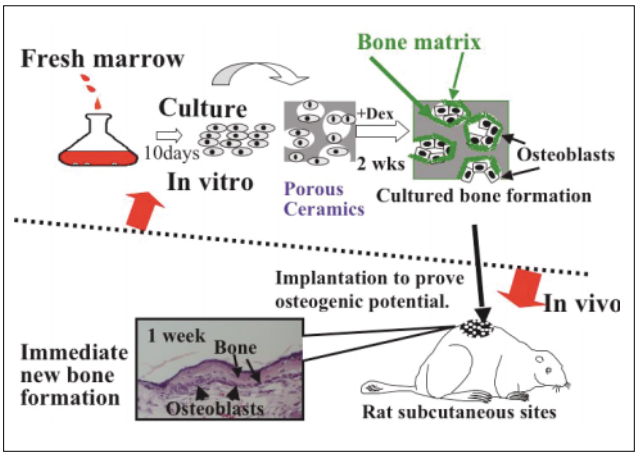

Fig.2 Fabrication of osteogenic materials using cultured bone

After culture expansion of marrow MSCs, the cells were combined with porous ceramics and further cultured in osteogenic medium for 2 weeks, when osteoblasts/bone matrix appeared in the pore areas of the ceramics (cultured bone formation). The cultured bone was implanted at rat subcutaneous sites. Even after about one week, new bone formation was detected inside the pore areas of the ceramics.

medium with 15% serum. Although fetal bovine serum is the golden standard in cell culture, we used the patient's own serum due to the risk of developing an illness such as bovine spongiform encephalopathy (BSE).

Detailed methods are as follows: We added the cells from three mL fresh aspirated bone marrow into two 75 cm^2 plastic culture flasks and cultured with changes of the medium at intervals of 3 times per week. At the time of the medium change, non-adherent hematopoietic cells were removed, leaving only adherent cells in the dish. After 10 to 11 days, the number of adherent cells grew and reached more than several million. The cells were collected after trypsinization (first passage) and further cultured in other flasks. After 4 to 8 days, the shape of most cells was fibroblastic. The cells were negative for hematopoietic markers (CD14, 34, 45) but positive for markers present in mesenchymal cells (CD13, 29, 90). These findings indicate that the adherent fibroblastic cells were mesenchymal types[17].

The adherent mesenchymal cells were trypsinized (second passage) and poured on the ceramics and cultured in the above medium supplemented with osteogenic factors; beta-glycerophosphate, vitamin C and dexamethasone (Dex) for 2 weeks. Calcium accumulation, alkaline phosphatase (ALP) activity were marked in the culture with Dex. The data indicated that the surface of the ceramic was covered with the patient's derived cultured osteoblasts/bone matrix (f cultured bone), which are implanted into the patients.

Many osteoarthritic and rheumatoid arthritic patients need total joint replacements; these prosthetic devices have problems including loosening of the implants. To prevent loosening, the prostheses are fabricated with porous structures or coated with bioactive materials such as HA. As we can alter the surface of materials using the tissue engineering approach with MSCs, we established a new concept to prevent such loosening, which is to coat joint prostheses with cultured bone[9]. In this approach, the second step of our approach is that the MSCs were seeded on alumina joint prostheses and cultured bone was formed on the prostheses. We started this tissue engineered approach in 2001 to prevent the loosening of the prostheses and the preliminary results were excellent[16]. We operated more than 50 patients with osteoarthritis, aseptic necrosis and bone tumor. Cultured bone was formed on porous triclcium phosphate ceramics and on hydroxyapatite ceramics for bone necrosis[18] and tumor patients[19], respectively. All the patients showed no serious side effects and many showed early bone healings around the implanted sites. However, some cases showed slow proliferation of MSCs and limited osteogenic differentiation.

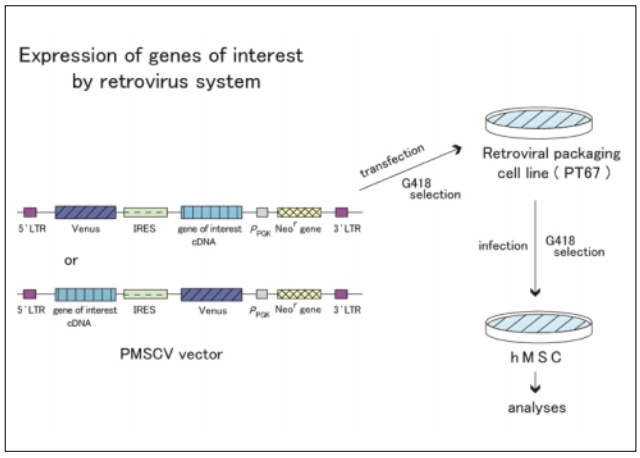

Fig.3 Schematic diagram of forced expression of genes of interest in human MSCs by retroviral system

Constructs were made for forced expression of genes of interest, in which *IRES* sequence was placed between the gene and *Venus*. Expression of target genes is driven by the LTR promoter, which is from the murine stem cell PCMV virus. Expression of the neomycin resistance gene is controlled by the murine phosphoglycerate kinase (PGK) promoter (P_{PGK}). After packaging cells, PT67, were transfected with a construct, the cells were cultured in the presence of G418 for antibiotic selection. Subsequently, human bone marrow MSCs were infected with supernatants derived from the PT67 cells. The MSCs were cultured with G418 for selection. From ref.26), with permission from Elsevier.

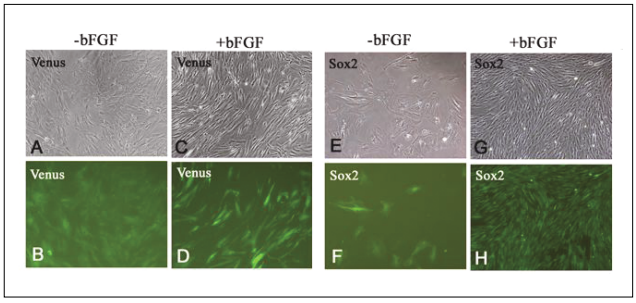

Fig.4 Distinct effects of addition of bFGF in culture media on morphology of Venus- and Sox2-expressing cells

Morphology of human MSCs (P5) that were infected with either construct, *Venus* (A-D) or *Venus-IRES-Sox2* (E-H). (A,C,E,G) phase-contrast images, (B,D,F,H) fluorescent images of the Venus protein. The cells (P5) were cultured in media containing 10%FBS (A-H). (A,B,E,F) No growth factors and (C,D,G) the bFGF protein (10ng/ml) was added in culture media. Distinct proliferation pattern and morphology of Venus- and Sox2-expressing cells are obvious. In the case of Venus-expressing cells, addition of bFGF in culture medium resulted in morphology changes, where the cells became elongated in shape (D). In contrast, Sox2-expressing cells grew well as small cells in the presence of bFGF(H). From ref.26), with permission from Elsevier.

MSCs could maintain high proliferation/differentiation capability by forced expression of transcripition factors

Murine embryonic stem cells (ES cells) are commonly maintained on primary mouse embryonic fibroblast feeder cells in culture medium supplemented with bovine serum and leukaemia inhibitory factor (LIF)[20]. In the absence of LIF, murine ES cells differentiate spontaneously in serum containing culture medium. 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 fibroblast growth factor (FGF) to maintain self renewal, pluripotency and prevent differentiation[21].

In addition to these factors, Oct4, Nanog and Sox2 are considered to form transcriptional regulatory circuitry for pluripotency and self-renewal of ES cells[22-24]. These observations demonstrate a possibility that forced expression of those transcription factors could render bone marrow MSCs better growth and plasticity properties. Especially, Sox2 is also known to be essential for neural stem cells as well[25]. We therefore introduced Sox2 gene into huma MSCs[26]. In order to achieve high efficiency of introduction and subsequent stable expression of the Sox2 in human bone marrow derived MSCs, we employed a retrovirus system (Fig.3). We used the construct in which IRES sequence was placed between the gene of interest (Sox2) and the *Venus* gene. We were able to detect the protein expression from each transgene exclusively in the nucleus in infected MSCs (Fig.4)[26].

Growth pattern of Sox2-expressing MSCs

There seemed to be essentially no difference between Sox2-expressing cells (P5) and control cells (P5), in which only the Venus protein was expressed, in terms of morphology and growth ability. We found, however, that Sox2-expressing cells showed distinct growth pattern in the presence of bFGF in culture media

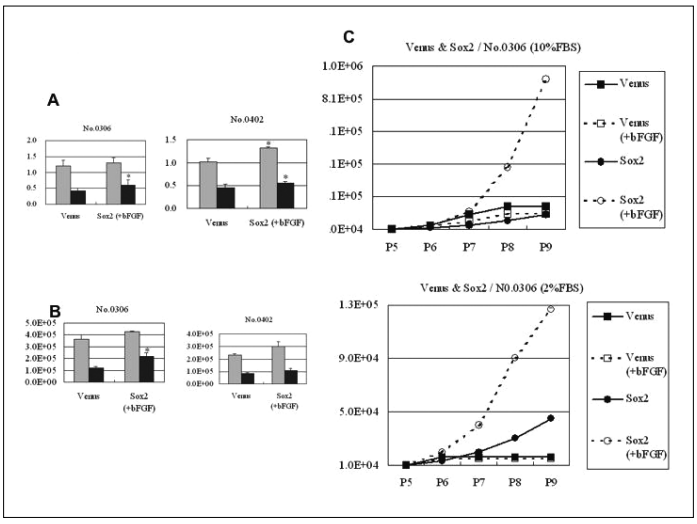

Fig.5 Proliferation activities of Venus- and Sox2-expressing MSCs

Proliferation activities of human MSCs infected with either construct, *Venus* or *Venus-IRES-Sox2*. (A,B) The cells were cultured in the presence of either 10% (light gray) or 1% (dark gray) FBS. Sox2-expressing cells were cultured in media containing the bFGF protein (10ng/ml). The results of WST assay (OD450nm) are shown in A (n=8), and the results of cell number count are in B (n=2). The cells (P5: No.0306 and No.0402) were seeded at 2 x 10^3 cells/well in an either 24- or 6-well plate. Measurement was done when the cells reached near confluence. Asterisk indicates a significant difference compared to the control (t-test: $p<0.05$). (C) MSCs were cultured in the presence of either 10% or 2% FBS, with or without addition of the bFGF protein (10ng/ml). The cells (cryopreserved P5: No.0306) were seeded at 1 x 10^4 cells/well in a 6-well plate, and the cell number was counted at every passage (4 times until P9) (n=3). The passage number of the cells is indicated. From ref.26), with permission from Elsevier.

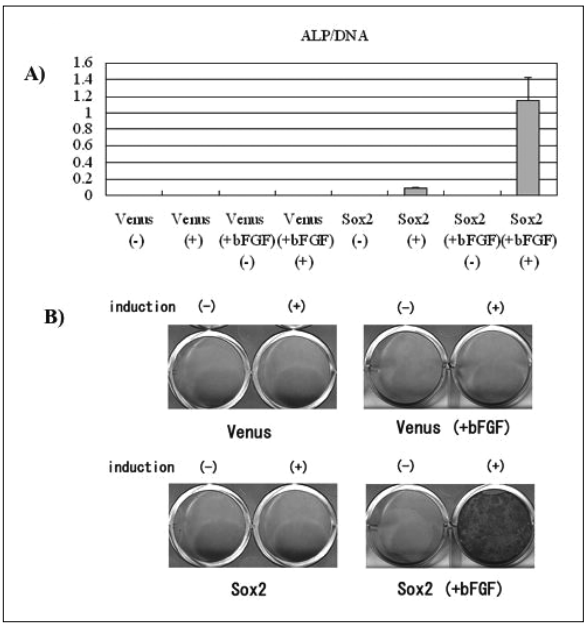

Fig.6 Osteogenic activities of Venus- and Sox2-expressing MSCs

(A,B) Osteogenic activities of human MSCs infected with either construct, *Venus* or *Sox2-IRES-Venus*. The results of ALP activity measurement (umol/ug) (A) and Alizarin Red S staining (B) are shown. The cells (P4: No.0305) were cultured in osteogenic differentiation media, with or without the addition of bFGF (10ng/ml) for 2 weeks (n=3). (-) control cells with no induction, (+) the cells with induction. Significant ALP activity (A) and strong Alizarin Red staining (B) were observed only in the case of the Sox2-expressing cells with bFGF. From ref.26), with permission from Elsevier.

(Fig.4). In the presence of the bFGF protein in culture media, human bone marrow MSCs show characteristic morphology changes, in which the cells become elongated in shape, and consequently take on more complex morphology with occasional cell protrusions (Fig.4C,D). Sox2-expressing cells did not show such morphology changes, however. The cells responded to bFGF very differently, where the cells grew well as relatively round and small cells in colonies (Fig.4G,H). We confirmed by FACS analysis that the Sox2-expressing cells were indeed relatively small in culture media containing bFGF. In general, the early passage MSCs show high expansion and differentiation potentials, and are small and simple in morphology. In this respect, it is interesting that Sox2-expressing cells cultured in media containing bFGF were small and simple in shape[26].

These observations are quite intriguing in the sense that bFGF is considered to be critical for self-renewal of Sox2-expressing stem cells such as neural stem cells and human ES cells. It has also been reported recently that in human embryonic carcinoma (EC) cells, the loss of self-renewal correlated with the down-regulation of genes involved in FGF signaling, suggesting FGF signaling is crucial for maintaining the undifferentiated state of human EC cells[27]. In the case of MSCs, however, bFGF has been shown to promote proliferation as well as differentiation for osteoblasts and neural cells[28]. Our observations suggest that bFGF signaling activation in the presence of Sox2 expression plays an important role in self-renewal of those stem cells. The response of Sox2-expressing cells to bFGF seems to be specific, because the similar growth pattern was not observed in the case

of either EGF or PDGF, both of which are commonly used growth factors for MSC culture[26].

Proliferation and differentiation potentials of Sox2-expressing MSCs

We evaluated growth ability of Sox2-expressing MSCs in both high and low serum conditions compared to control cells in which only Venus was expressed. We found in both conditions that Sox2-expressing cells in the presence of bFGF had higher proliferation potential than control cells (Fig.5A,B). We next conducted cell growth assay by counting cell number for several passages, and found that Sox2-expressing MSCs with bFGF showed significantly higher cell growth than control cells particularly in late passages and in a low serum condition (Fig.5C)[26].

We next examined differentiation potential for osteoblasts of Sox2-expressing cells, and found that Sox2-expressing cells showed higher osteogenic potential than control cells in terms of both ALP activity and calcium deposition assayed by Alizarin Red staining in the presence of bFGF (Fig.6A,B)[26]. These observations indicate that Sox2-expressing cells in media containing bFGF maintained capabilities for proliferation as well as responding to glucocorticoid hormone dexamethasone resulting in osteogenic differentiation. Thus, the cells have self-renewal and differentiation capabilities.

Conclusive remarks

It has recently been reported by Takahashi and Yamanaka at Kyoto University that induced pluripotent stem cells (iPS) can be directly generated from mouse[29] and human[30] fibroblasts by the introduction of concomitant four defined genes, one of which was *Sox2*. Although Nanog was not one of the four genes[28,29], its functional importance was evident from the fact that up-regulation of the Nanog gene seemed to be critical for reprogramming the cells[31] and University of Wisconsin reported the generation of iPS using four genes in whith Nanog was included[32]. Furthermore, we also found that Nanog-expressing cells showed higher differentiation abilities for osteoblasts than control cells in terms of both ALP activity and calcium deposition assayed by Alizarin Red staining[26]. It is interesting to note that addition of bFGF had adverse effects on osteogenesis for Nanog-expressing cells in contrast to Sox2-expressing cells. Although the generation of iPS indicate that the introduction of one of those transcription factors would not be sufficient to reprogram the genome, the functional importance of Sox2 and Nanog for altering the cell status was clearly demonstrated.

Our observations on the forced expression of Sox2 or Nanog in adult MSCs are indeed consistent with this notion 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 do 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; therefore our approach using single gene introduction[26] could be an effective and realistic way of maintaining high quality of MSCs for regenerative medicine, especially for bone tissue regeneration.

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