

# Chapter 16

## Bone Related Cell-Stimulating Scaffold Materials and a 3D Cellular Construct for Hard Tissue Regeneration

Osamu Suzuki and Takahisa Anada

**Abstract** The present article summarizes recently developed calcium phosphate-based scaffold materials that are capable of stimulating various cells involved in hard tissue formation as well as a method using newly developed cell culture devices to generate three dimensional (3D) cellular constructs. Synthetic octacalcium phosphate stimulates cellular functions of several cell types, including osteoblasts, chondrocytes, and pulp cells. The culture device promotes the easy assembly of chondrocytes in a 3D cellular construct. The scaffold materials, cellular constructs, and composite presented here are good candidates as implantable materials for the regeneration of hard tissue in bone defects depending on the severity of the tissue loss.

**Keywords** Octacalcium phosphate · Scaffold · Osteoblast · Spheroid · Polydimethylsiloxane · Cell culture device · Chondrocyte

### 16.1 Introduction

Bone tissue consists of cells related to bone formation and associated matrix materials, including collagen fibers mineralized with biomineral hydroxyapatite (HA) crystals and various growth factors [1]. Osteoblasts, osteoclasts, chondrocytes, endothelial cells, and bone marrow cells are involved in the remodeling processes that occur in bone tissues [1]. Recently, increased attention has been placed on developing three-dimensional (3D) scaffolds inoculated with exogenous cells or cell constructs without scaffold materials under well-controlled conditions that are suitable for increasing cell activity [2]. In this article, we summarize our methodology for constructing scaffolds particularly with synthetic biominerals, including scaffolds

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that can stimulate exogenous cellular activity as well as 3D cellular constructs, both of which could be implantable. These approaches may be effective therapies for bone regeneration of large defects caused by various diseases, such as trauma or tumors, due to the limited autologous bone available in these patients [3]. The development of an implantable artificial scaffold and cellular construct that has biological performance comparable to autologous bone is the current goal in the field of bone tissue engineering [4, 5]. We show here that octacalcium phosphate (OCP) is a suitable synthetic biomineral analogue [6, 7] and that a polydimethylsiloxane (PDMS)-based cell culture device is a useful tool for not only developing the appropriate scaffold for growing the cell construct, but also developing a construct of the bone forming-related cells, chondrocytes [8].

## 16.2 Performance of Synthetic Biomaterials and the Device Used for Cellular Construct Development

We have developed biomaterials and cell culture devices to analyze cell-materials and cell-cell interactions for the regeneration of hard tissues [8–10]. Our previous studies showed that the interaction between calcium phosphate materials, in particular OCP, and various cells involved in hard tissue formation stimulates cellular functions such as proliferation and differentiation [11–15]. Based on the evidence, we hypothesized that the combination of calcium phosphate materials and 3D cellular constructs improves their cellular activities and leads to effective regeneration of hard tissues [4, 9, 16]. In this review we describe the development of composite materials as scaffolds for bone tissue regeneration and the fabrication of devices for the construction of 3D cellular aggregates [8, 9, 17].

We previously elucidated the effects of OCP on cell proliferation and differentiation in various cell types. Table 16.1 shows the summary of the cell-materials interaction investigated to date:

- OCP promotes an early stage of osteoblastic differentiation of mouse bone marrow derived stromal ST-2 cells [11].
- OCP promotes osteoclastic differentiation of mouse bone marrow cells by increasing receptor activator of NF- $\kappa$ B ligand (RANKL) expression by osteoblasts [14].
- OCP promotes odontoblastic differentiation of rat pulp cells [15].
- OCP suppresses chondrogenic differentiation of mouse chondrogenic ATDC5 cells [12].
- OCP stimulates mesenchymal cells (MSCs) to enhance new bone formation in rat critical-sized defects if implanted with OCP and collagen scaffold [4].

**Table 16.1** Effect of OCP crystals on cellular activities of calcified tissue derived cells.

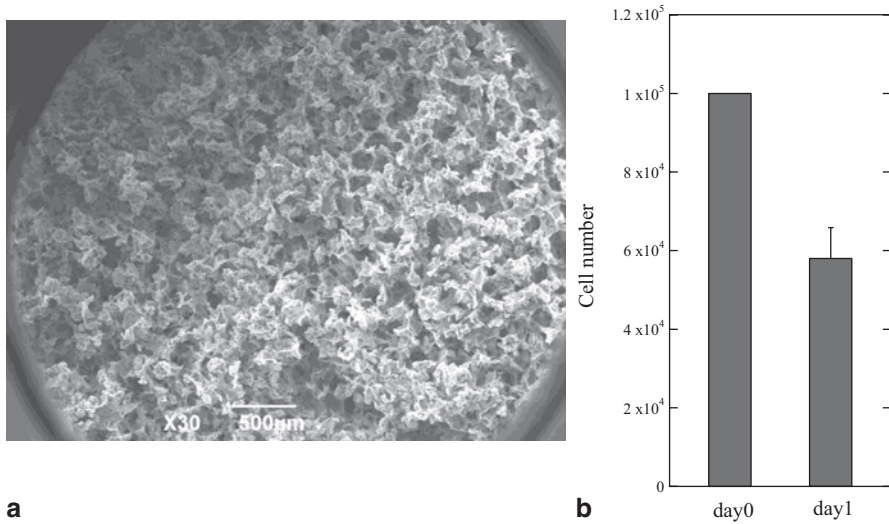
| Cells examined                             | Cellular response  | mRNA expression   | References |
|--|--|---|------------|
| Mouse bone marrow stromal ST-2 cells       | Osteoblastic differentiation                                     | Up-regulation of ALP <sup>a</sup> , osterix and Col I       | [11, 13]   |
| Mouse bone marrow cells/osteoblastic cells | Osteoclast formation (in the absence of vitamin D <sub>3</sub> ) | Up-regulation of RANKL <sup>b</sup> in osteoblasts          | [14]       |
| Rat dental pulp cells                      | Odontoblastic differentiation                                    | Up-regulation of DSPP <sup>c</sup> , DMP <sup>d</sup> , ALP | [15]       |
| Chondrogenic ATDC5 cells                   | Inhibition of chondrogenic differentiation                       | Down-regulation of Sox6 <sup>e</sup>                        | [12]       |

<sup>a</sup>Alkaline phosphatase; <sup>b</sup>Receptor activator of nuclear factor-kappa B ligand; <sup>c</sup>Dentin sialophosphoprotein; <sup>d</sup>Dentin matrix protein; <sup>e</sup>SRY (sex determining region Y)-box 6

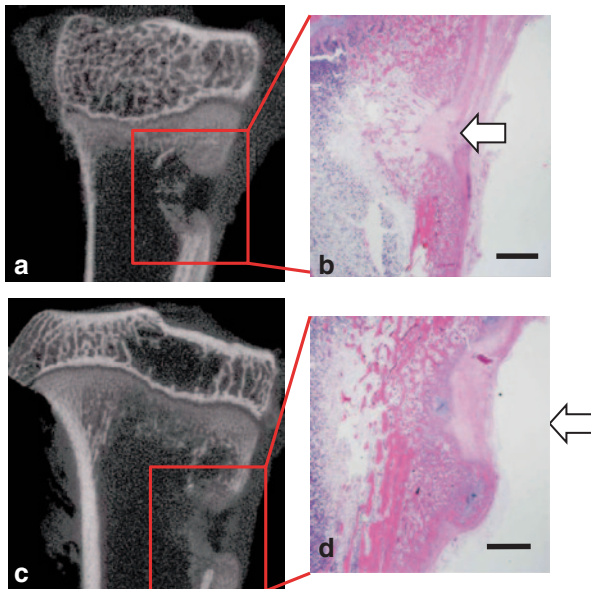
## 16.3 Development of the Scaffold Materials for Tissue Regeneration and Inoculated Cells

### 16.3.1 *Effect of Co-Precipitation of OCP With Gelatin Molecules on Their Composite Scaffolds [9]*

The details for material preparation have been reported elsewhere [9]. Osteoblastic cells should interact with the co-precipitated OCP crystals as much as possible in order to maximize the cellular activity. To do so, OCP crystals were designed to be homogeneously dispersed in a gelatin (Gel) matrix. OCP-dispersed gelatin composite (OCP/Gel) was obtained through the co-precipitation of OCP from calcium and phosphate solutions under a supersaturated condition with respect to HA and OCP and at constant pH and temperature. OCP/Gel precipitates were molded, lyophilized, and treated dehydrothermally for the cross-linking step. The resultant materials consisted of highly porous (over 90% in porosity) 3D structure and contained 40% of OCP crystals by weight. The cellular adhesive property was examined using osteoblastic ST-2 cells inoculated onto OCP/Gel composites. Mouse bone marrow stromal ST-2 cells were seeded onto the OCP/Gel disks and the number of the viable cells attached was measured at 1 day post-inoculation. More than 60–70% of the seeded cells were attached to the disk of OCP/Gel composites (Fig. 16.1), suggesting that host osteoblastic cells can attach, proliferate, and differentiate on OCP/Gel composite if implanted in a bone defect [9]. In fact, when the disks of OCP/Gel composite were implanted in rat critical-sized 9 mm diameter defects, new bone formation was observed together with accelerated biodegradation of the OCP/Gel composite by osteoclastic resorption [9]. Furthermore, the OCP/Gel composite was able to repair tibia bone defects of 5 mm in diameter, even in the early stages of implantation, accompanied by the relatively rapid material degradation and active new bone formation (Fig. 16.2). These results suggest that OCP and Gel can be successfully used as exogenous cellular scaffold materials.

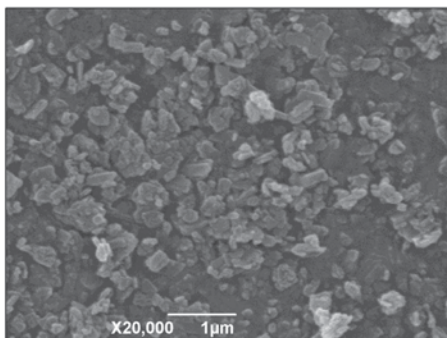


**Fig. 16.1** **a** Scanning electron micrograph of OCP40/Gel composite. **b** Cell attachment to OCP40/Gel composite after 1 day of incubation. Reproduced from [9] with some modification with permission from Elsevier



**Fig. 16.2** Coronal view of micro CT and tissue section from rabbit tibia that was stained with hematoxylin and eosin. Control group (a), (b) and OCP40/Gel implantation group (c) and (d) at 2 weeks post-operation. Arrows: the site of the bone defect and OCP/Gel implantation. Bars: 3 mm. Reproduced from [17] with some modification with permission from Japanese Association of Inorganic Phosphorous Chemistry

**Fig. 16.3** Scanning electron micrograph of nano-submicro OCP crystals. Bar = 1  $\mu\text{m}$



### ***16.3.2 The Effect Of Micro-Nano OCP Crystal Inclusion in Gel Matrix [10]***

A previous study reported that the microstructure of OCP crystals may be one factor that controls their bone regenerative properties when implanted in critical-sized mouse calvaria [18]. It has been shown that crystals that are several micrometers in length induce significantly greater enhancement of bone regeneration than OCP crystals greater than 10  $\mu\text{m}$  in length [19]. Based on the experimental evidence, we investigated the effect of OCP crystals mechanically ground into micro-nano sized crystals dispersed in Gel matrix on bone regeneration if implanted in rat critical-sized calvaria defects [10]. The micro-nano OCP/Gel composite sufficiently provided a scaffold to support host osteoblastic cells as the material provided osteoconductivity in the defect [10]. Figure 16.3 shows the scanning electron micrograph of the OCP crystals ground mechanically. The micro-nano crystals were included together with larger OCP crystals. In fact, when the size of the particles was analyzed by dynamic light scattering in ethanol, it was found that the size was approximately 520 nm although there was a second distribution of approximately 24  $\mu\text{m}$  [10].

### ***16.3.3 Effect of OCP Crystals on MSC Differentiation by the OCP/Col/MSC Composite [4]***

The effect of OCP crystals on differentiation of rat bone marrow-derived mesenchymal stem cells (MSCs) in collagen matrix (Col) was investigated by implanting OCP/Col/MSC in rat critical-sized calvaria defects [4]. Alkaline phosphatase (ALP)-expressing MSCs were obtained by incubating in osteogenic medium containing basic FGF, and then the cells were inoculated on OCP/Col and implanted in rat critical-sized calvaria defects. Eight weeks post-implantation, bone regeneration increased in the following order: Col, OCP/Col, and OCP/Col/MSCs. These results suggest that OCP crystals can enhance the differentiation of MSCs within an OCP/Col matrix in vivo [4].

### ***16.3.4 Possible Scaffold Materials For Osteoblastic Cells***

In order to develop osteoblastic cells for bone tissue regeneration, scaffold materials such as OCP crystals may effectively support cellular differentiation. The advancement of mineralization by this material under physiological conditions may control osteoblastic differentiation. Therefore, future studies will focus on designing scaffold materials for optimal distribution of cells and to determine which type of cells should be included for repair of the targeted hard tissues.

## **16.4 Spheroid Cell Culture Devices And Application With Chondrocytes**

### ***16.4.1 3D Cellular Constructs***

Several studies have described the preparation of 3D cellular constructs [20]. Among them, much attention has focused on fabricating the cellular aggregates to have a spheroid shape that can act as building blocks for a large number of the cells [2]. It is thought that as the aggregate size increases, the spheroids begin to mimic the functions of living tissues compared to monolayer cell cultures regarding the morphology, cell-cell interactions, cell-matrix interactions, and signaling. This effect may be due to the 3D contact and close association of the cells with each other. Indeed, hepatocytes cultured in 3D exhibit long-term liver-specific functions [21–23]. The formation of osteoblast spheroids increases the expression of several proteins involved in bone formation [24]. It is well known that endochondral bone ossification initiates nodule formation with the aggregation of mesenchymal cells. The self-assembled mesenchymal cell aggregates cause dense cell-cell interactions and subsequent differentiation into chondrocytes [25]. A previous study described an approach to simulate mesenchymal condensation and cellular interactions, and mesenchymal stem cells (MSCs) or chondrocytes have been examined for forming the pellet or spheroid for chondrogenesis. Moreover, spheroids have been verified as biological models in investigations of microtumors and metastases [26]. Moreover, spheroids have been used as drug screening assays as an alternative to animal models [27].

Several techniques for the culture of spheroids have been reported, including cultivation on nonadherent surfaces [28], cultivation in spinner flasks [29], the hanging drop method [30], micromolding technique [31], and a spheroid array on a micropatterned surface [32, 33]. However, conventional techniques are difficult to produce large amount of spheroids, control the spheroid size, and immobilize spheroids on the culture surface, which results in the requirement for invasive collection of cells.

### 16.4.2 Spheroid Culture Device Utilizing Thin Membrane Deformation Under Decompression (Prototype Device) [34]

We have previously developed a spheroid culture device to overcome the limitations of conventional methods through the utilization of thin membrane deformation by decreasing the pressure [34]. This culture device can be constructed with thousands of milli- to micro-sized hemispherical cavities by deforming a thin polydimethylsiloxane (PDMS) membrane under decompression. The resulting device can rapidly produce many spheroids at the same time within the hemispherical multicavities and can control the diameter of the spheroids within a narrow distribution. This approach also allows for the collection of spheroids from the device using a non-invasive approach. The device is constructed from PDMS, which is an acrylic resin plate, and a glass plate (Fig. 16.4). The PDMS chamber and membrane are assembled as a cell culture chamber. Figure 16.5 shows the mechanism of action of

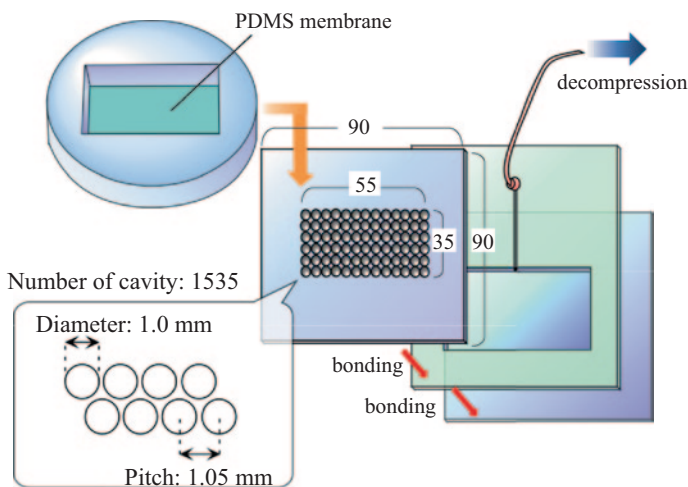


Fig. 16.4 Schematic illustration of components of the spheroid culture device. Redrawn from [34] with permission from Elsevier

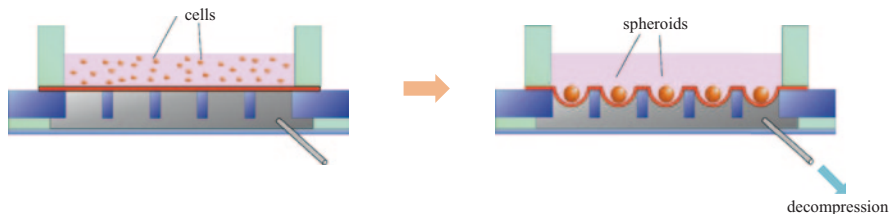


Fig. 16.5 Schematic illustration of the mechanism of action of the device. Modified from [34] with permission from Elsevier



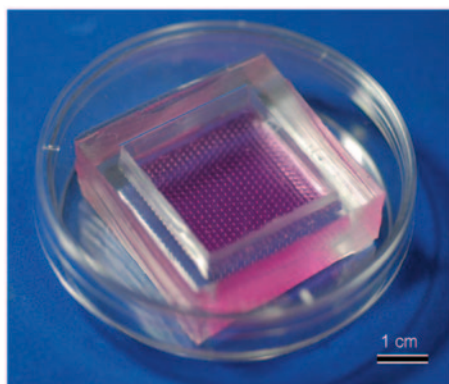
the device. The multicavities in the acrylic resin plate are covered by the PDMS membrane of the culture chamber. These multicavities are then connected with a silicon tube to reduce pressure. Thus, the PDMS membrane can be deformed as a hemispherical cavity. A Pluronic F-127 solution is added to the culture chamber to prevent cell attachment and promote spheroid formation. The cell suspension is then added into the cell culture chamber, and a thin PDMS membrane is deformed by pressure reduction. The cells on the PDMS membrane self-assemble, which results in spheroid formation in the hemispherical cavity.

To test this device, human osteosarcoma MG63 cells and the human hepatoma cell line HepG2 were cultured in the cavities. We found that the cells spontaneously dropped into the cavities and a single spheroid formed in each. At the end of the culture, the cells were easily collected from the culture device by pressure release. Importantly, the recovery of cells from the device without enzymatic treatment provides a great advantage in applications for regenerative medicine.

### 16.4.3 Spheroid Formation Utilizing a PDMS-Based Culture Chip [8]

Our described spheroid culture device utilizes thin PDMS membrane deformation through negative pressure, but the decompression requires use of an external vacuum pump throughout the culture time in order to maintain the membrane deformation. In addition, the fabrication process is relatively time consuming. Therefore, we improved this procedure by using the prototype device as a master mold with a simple and rapid lithography technique [34]. Briefly, a PDMS prepolymer was poured into the PDMS negative mold and cured. The PDMS replica was peeled off from the mold and then used in the cell culture. To prevent cell adhesion to the PDMS surface and accelerate the formation of spheroids, the surface of the PDMS chip was covalently modified with polyethyleneglycol diacrylate (PEGDA) [35]. The culture chips were inlaid into a 6 cm diameter polystyrene dish to prevent contamination of the cultured cells (Fig. 16.6). The chips were designed to be com-

**Fig. 16.6** Photograph of PDMS-based spheroid culture chip. Reproduced from [8] with permission from Fuji Technology Press





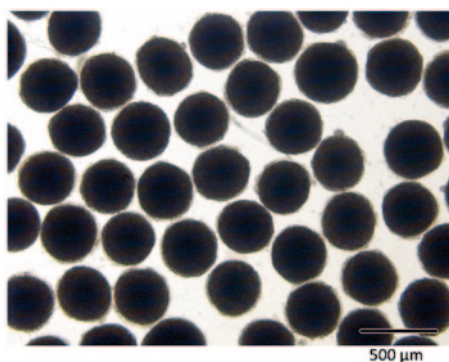
prised of multicavities (512 wells, 1.0 mm in diameter, 1.05 mm pitch, and 1060  $\mu\text{m}$  in depth) on a 25  $\times$  25 mm section of the cell culture area.

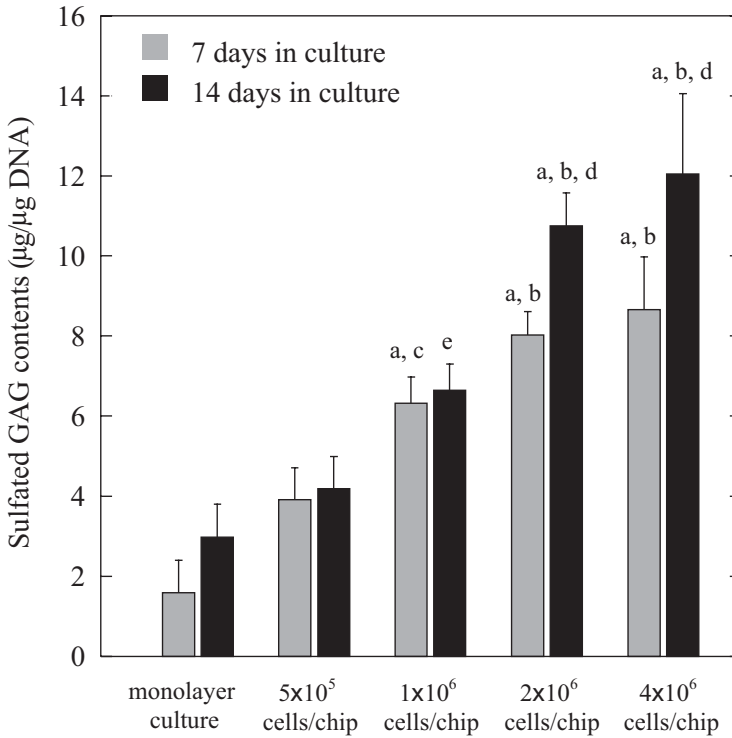
#### 16.4.4 Chondrocyte Culture Using a PDMS-Based Culture Chip

It has been reported that the size and shape of the cell aggregates are important for cell proliferation and differentiation. Numerous studies have reported that dense cell pellets consisting of an aggregate of MSCs or chondrocytes enhance the expression of cartilage-specific genes, secretion of extracellular matrix such as glycosaminoglycan (GAG) and type II collagen, and development of cartilage-like morphology [36].

The murine embryonic carcinoma cell line ATDC5 was previously shown to differentiate into chondrocytes from mesenchymal condensation in the presence of insulin [37]. ATDC5 cells at different concentrations ( $5 \times 10^5$ ,  $1 \times 10^6$ ,  $2 \times 10^6$ , and  $4 \times 10^6$  cells/chip) were added to the chips and grown in chondrogenic medium. Cells spontaneously dropped into the cavities, and a single spheroid formed in each within 3 h. In addition, the spheroid diameters increased depending on the initial cell density. Cells were collected from the culture chip by washing them out with PBS or culture medium without enzymatic treatment at the end of the culture period. The culture chip produced well-rounded spheroids with narrow size distributions (Fig. 16.7). The secretion of sulfated GAG by ATDC5 cells tended to increase with time in all samples (Fig. 16.8). Moreover, sulfated GAG secretion in the spheroid culture system was higher than that in the monolayer culture and increased as the initial cell density increased. After 14 days of culture, the cells seeded at a density of  $4 \times 10^6$  cells/chip had the greatest secretion (4.0-fold higher than that in the monolayer culture). A higher cell seeding density significantly improved the differentiation of ATDC5 cells in the spheroid culture chip. Further studies are required to elucidate the mechanism that facilitates the chondrogenic phenotype of ATDC5 cells by assessing the culture chip at the molecular level, including changes in the gene expression level during chondrogenic differentiation. Some groups have

**Fig. 16.7** Light microscopic image of ATDC5 spheroids collected from the culture chip on day 14 of culture. Reproduced from [8] with permission from Fuji Technology Press





**Fig. 16.8** Changes in sulfated GAG content of ATDC5 cells normalized by DNA. <sup>a</sup> $p < 0.01$  compared to monolayer culture. <sup>b</sup> $p < 0.01$  compared to  $5 \times 10^5$  cells/chip. <sup>c</sup> $p < 0.05$  compared to  $5 \times 10^5$  cells/chip. <sup>d</sup> $p < 0.01$  compared to  $1 \times 10^6$  cells/chip. <sup>e</sup> $p < 0.05$  compared to monolayer culture. Reproduced from [8] with permission from Fuji Technology Press

reported on the regeneration of cartilage using chondrocyte spheroids injected without scaffold material [38–40] or with hydrogel [41]. Our system could be useful for the mass production of transplantable spheroids, and we believe that this culture system can be potentially used for a wide variety of tissue engineering strategies.

## 16.5 Conclusion

The present studies underscore the effectiveness of the scaffold material, and in particular that of OCP crystals, to enhance the activity of calcified tissue-relating cells such as osteoblasts. Furthermore, the device used for cellular construct preparation effectively formed 3D spheroids and facilitates cellular activity, especially chondrocytes. Further studies are underway to establish the effect of the interaction between the scaffold materials and the cellular construct on cellular function, such as differentiation, and may be used to efficiently repair tissue.

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