Mesenchymal stem cells (MSCs) have multidifferentiation potential [2–4], and their most notable phenotypic characteristics are their in vitro cartilage and bone differentiation abilities. The choice of chondrogenic or osteogenic lineage of MSCs in vitro is decided by the culture conditions. When the same MSCs are embedded in the same scaffolds, their lineages depend on the composition of culture medium.

In this study, bone marrow MSCs were embedded in collagen gel [5–8] and cultured in chondrogenic or osteogenic medium. We compared morphologies of MSCs during chondrogenic [9–11] and osteogenic [12–14] differentiation sequentially. Our detailed morphological investigation clarified some novel characteristics of MSCs during chondrogenesis and osteogenesis, and this simple comparative model will be helpful for understanding cartilage and bone formation.

Materials and methods

Human bone marrow mesenchymal stem cell culture

Bone marrow MSCs derived from a healthy female donor, a Caucasian aged 23 years, were purchased from Lonza Walkersville (Walkersville, MD, USA). Passage 2 cells were proliferated with mesenchymal stem cell growth medium (Lonza Walkersville) containing 10 % fetal bovine serum and antibiotics at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Passage 3 cells were further proliferated, and passage 4 cells were used for the following analyses [15].

MSC/collagen composite

Collagen gel (Atelocollagen, 3 % type I collagen; Koken, Tokyo, Japan) in a 1-ml syringe was mixed thoroughly with the same volume of cell suspension medium contained in a similar connected 1-ml syringe at room temperature. To prepare the MSC/collagen composite at 10^8 cells/ml, 500 μl medium containing 10^8 cells was mixed with 500 μl atelocollagen gel. The MSC/collagen composites were then injected into 24-well culture plates (Falcon; Becton–Dickinson, Oxnard, CA, USA) to make discs 7 mm in diameter, and then incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 15 min to increase their firmness. Then, 1.5 ml medium was placed into each well and incubated for 21 days. The medium was replaced twice a week [16].

Chondrogenesis

The chondrogenesis induction medium was high-glucose Dulbecco's modified Eagle's medium containing 10 ng/ml transforming growth factor-β3 (R&D Systems, Minneapolis, MN, USA) in addition to 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin (BioWhittaker).

Osteogenesis

The osteogenesis induction medium was mesenchymal stem cell growth medium with 0.1 μM dexamethasone, 50 μg/ml ascorbate-2-phosphate, and 10 mM β-glycerol phosphate.

Histology

The cultures were ended by fixing the composites with 2.5 % glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 2 h. The composites were washed overnight at 4 °C in the same buffer and postfixed with 1 % OsO_4 buffered with 0.1 M PBS for 2 h. The composites were then dehydrated in a graded series of ethanol and embedded in Epon 812. Semithin (1 μm) sections for light microscopy were collected on glass slides and stained for 30 s with toluidine blue.

Transmission electron microscopy

Ultrathin (90 nm) sections were collected on copper grids, double stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (TEM) (H-7100; Hitachi, Hitachinaka, Japan). The electron diffraction method was performed on the selected area. The d-spacings of the diffraction patterns were calibrated using the d-spacings of gold determined under identical conditions [17].

Immunohistochemistry

Immediately after fixation with 4 % paraformaldehyde in 0.1 M PBS for 1 h, the composites were immersed in 25 % sucrose in 0.1 M PBS for 24 h at 4 °C, mounted in *ortho*-chlorotoluene (OCT) embedding medium, and quickly frozen in liquid nitrogen. Then, 6-μm frozen sections were cut on a CM1900 cryostat (Reichert, Vienna, Austria) at a temperature of −15 °C. The frozen sections were placed on silane-coated glass slides and washed in 0.1 M PBS. For the immunohistochemistry, the sections were blocked with 0.1 M PBS containing 1 % normal goat serum for 1 h at 25 °C. Mouse antibodies against collagen type I, collagen type II (Daiichi Fine Chemical, Takaoka, Japan), and chondroitin sulfate-proteoglycan (Seikagaku kogyo, Tokyo,

Japan) and rabbit antibodies against osteocalcin, osteopontin (LSL, Tokyo, Japan), and connexin 43 (Zymed, South San Francisco, CA, USA) in 1 % bovine serum albumin (BSA) were incubated with the sections for 24 h at 4 °C. These antibodies were diluted at 1:100–1:500 in 0.1 M PBS. After extensive washing with 0.1 M PBS, the sections were incubated for 30 min with biotinylated secondary antibodies. Immunohistochemistry was detected with a Zymed Histostain kit (Zymed). Counterstaining was performed with methyl green.

Immunoelectron microscopy

The sections were placed on silane-coated glass slides and washed in 0.1 M PBS. For immunoelectron microscopy, the frozen sections were blocked with 0.1 M PBS containing 1 % normal goat serum for 1 h at 4 °C. Mouse antibody against collagen type I (Daiichi Fine Chemical) was added at a dilution of 1:50 for 24 h at 4 °C. The sections were then washed with 0.1 M PBS and incubated with goat anti-mouse IgG + IgM conjugated with 15-nm gold colloidal particles (dilution 1:20; British Bio Cell International, Golden Gate, UK) for 24 h at 4 °C. After incubation, the sections were washed with 0.1 M PBS for 30 min. The sections were subsequently fixed in 2.5 % glutaraldehyde and postfixed in a 1 % OsO_4 solution in 0.1 M PBS before being dehydrated in a graded series of ethanol and dried in a critical point drying apparatus (HCP-2; Hitachi) with liquid CO_2. Next, the sections were spatter-coated with osmium using NL-OPC80N (Filgen, Nagoya, Japan) and examined by scanning electron microscopy (SEM) (S-4500; Hitachi) and using an yttrium–aluminum–garnet (YAG) back-scattered detector (Hitachi) [18].

SEM–EDS analysis

SEM samples for immunoelectron microscopy were examined by SEM (S-4500; Hitachi) and analyzed by energy-dispersive X-ray spectroscopy (EDS) (EMAX-7000; Horiba, Kyoto, Japan). The EDS analysis involved characteristic X-ray lines, namely, Ca-kα and P-kα [19].

Results

MSC/collagen composite before induction

Before induction, the MSCs were dispersed and round in shape in the collagen gel (Fig. 1a). Transmission electron microscopy (TEM) demonstrated that the cells appeared to be stationary in the collagen gel. The collagen gel was homogeneous and not fibriform (Fig. 1b). The cell nuclei were notched, and the MSC contained mitochondria, endoplasmic reticulum, and Golgi apparatus (Fig. 1c). Immunoelectron microscopy showed only a few localizations of collagen type I, indicated by gold particles (Fig. 1d).

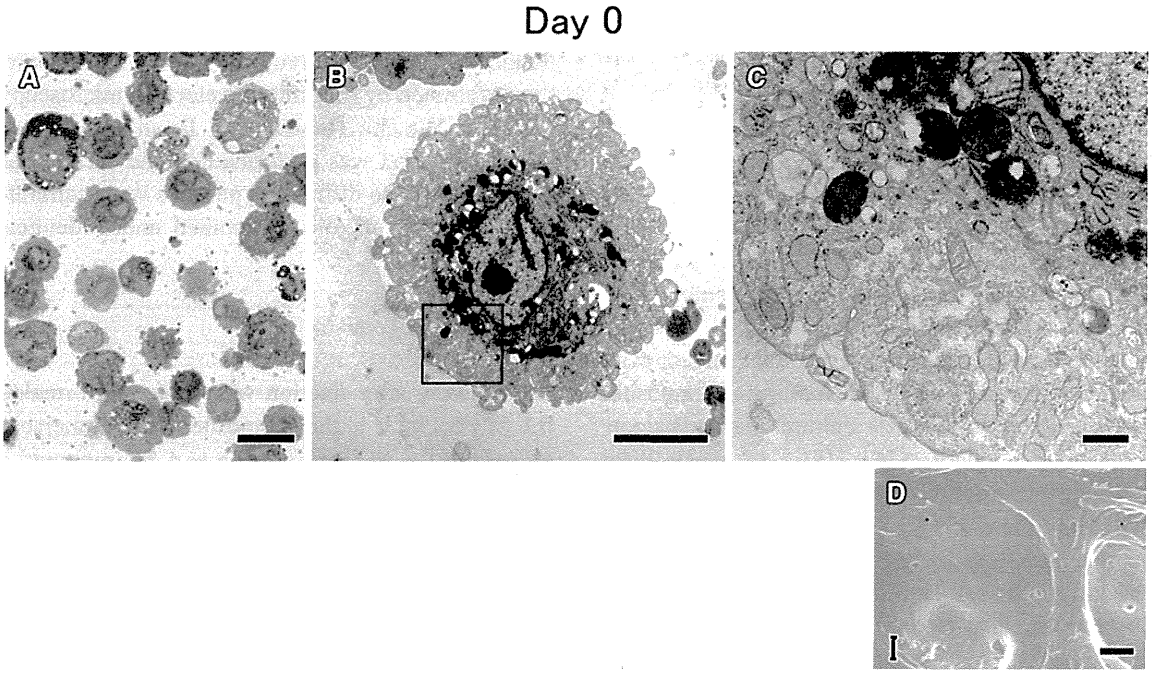

Fig. 1 Mesenchymal stem cell (MSC)/collagen composite before chondrogenic and osteogenic induction. **a** Light micrograph of composite stained with toluidine blue. **b** Transmission electron microscopy (TEM) image of MSC. **c** Higher magnification of the area indicated by the *square* in **b**. **d** Immunoelectron microscopic image taken by scanning electron microscopy (SEM) of collagen type I. *Bars* **a** 20 μm, **b** 5 μm, **c** 500 nm, **d** 200 nm

MSC/collagen composite at day 1

One day later, morphological differences were not seen between chondrogenesis and osteogenesis. Most MSCs were still round but some had changed in shape (Fig. 2a, h). Cells with a large number of short processes (Fig. 2b, i) produced extracellular fibers (Fig. 2c, j). Immunohistochemically, collagen type I was strongly stained extracellularly (Fig. 2e, l), while collagen type II during chondrogenic induction (Fig. 2f) and osteocalcin during osteogenic induction (Fig. 2m) were hardly detected. The expression of collagen type I was confirmed adjacent to the MSC by immunoelectron microscopy (Fig. 2g, n).

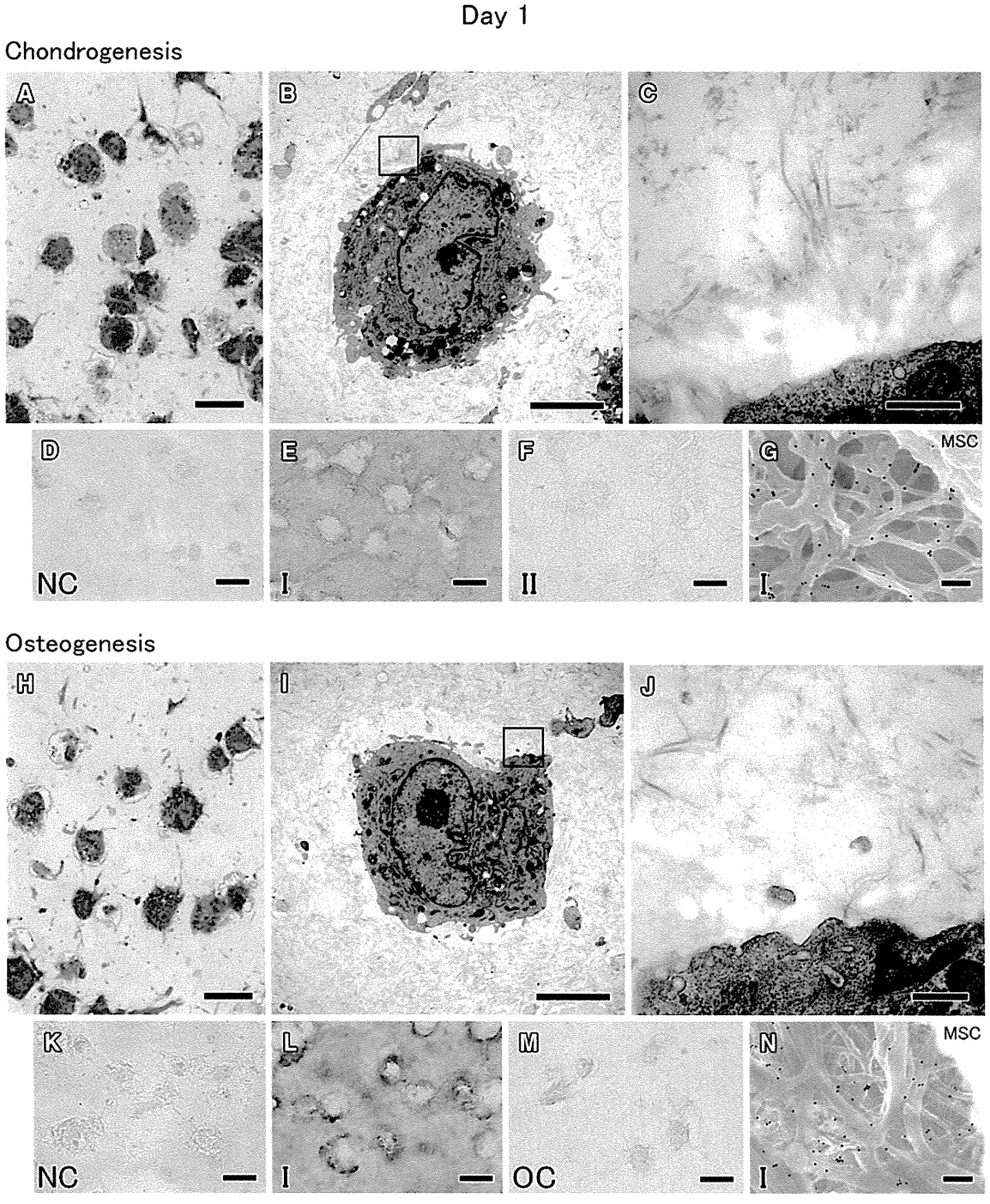

Fig. 2 MSC/collagen composite 1 day after chondrogenic (**a–g**) and osteogenic (**h–n**) induction. **a**, **h** Light micrographs of the composite stained with toluidine blue. **b**, **i** TEM images of MSC. **c**, **j** Higher magnifications of the areas indicated by the *squares* in **b** and **i**, respectively. **d–f**, **k–m** Immunohistochemistry of negative control (*NC*) (**d**, **k**), collagen type *I* (**e**, **l**), collagen type *II* (**f**), and osteocalcin (*OC*) (**m**). **g**, **n** Immunoelectron microscopic images of collagen type I. *Bars* **a**, **h** 20 μm; **b**, **i** 5 μm; **c**, **j** 500 nm; **d–f**, **k–m** 20 μm; **g**, **n** 200 nm

Cell–cell interaction

At day 3, although morphological features still appeared to be the same between chondrogenesis and osteogenesis, the shape of MSCs changed from round to elongated, and MSCs contained long processes (Fig. 3a, f). Gap junctions were observed at the surface of the MSCs (Fig. 3b, c, g, h). Immunohistochemistry demonstrated expression of connexin 43 (Fig. 3d, e, i, j), a marker for gap junctions.

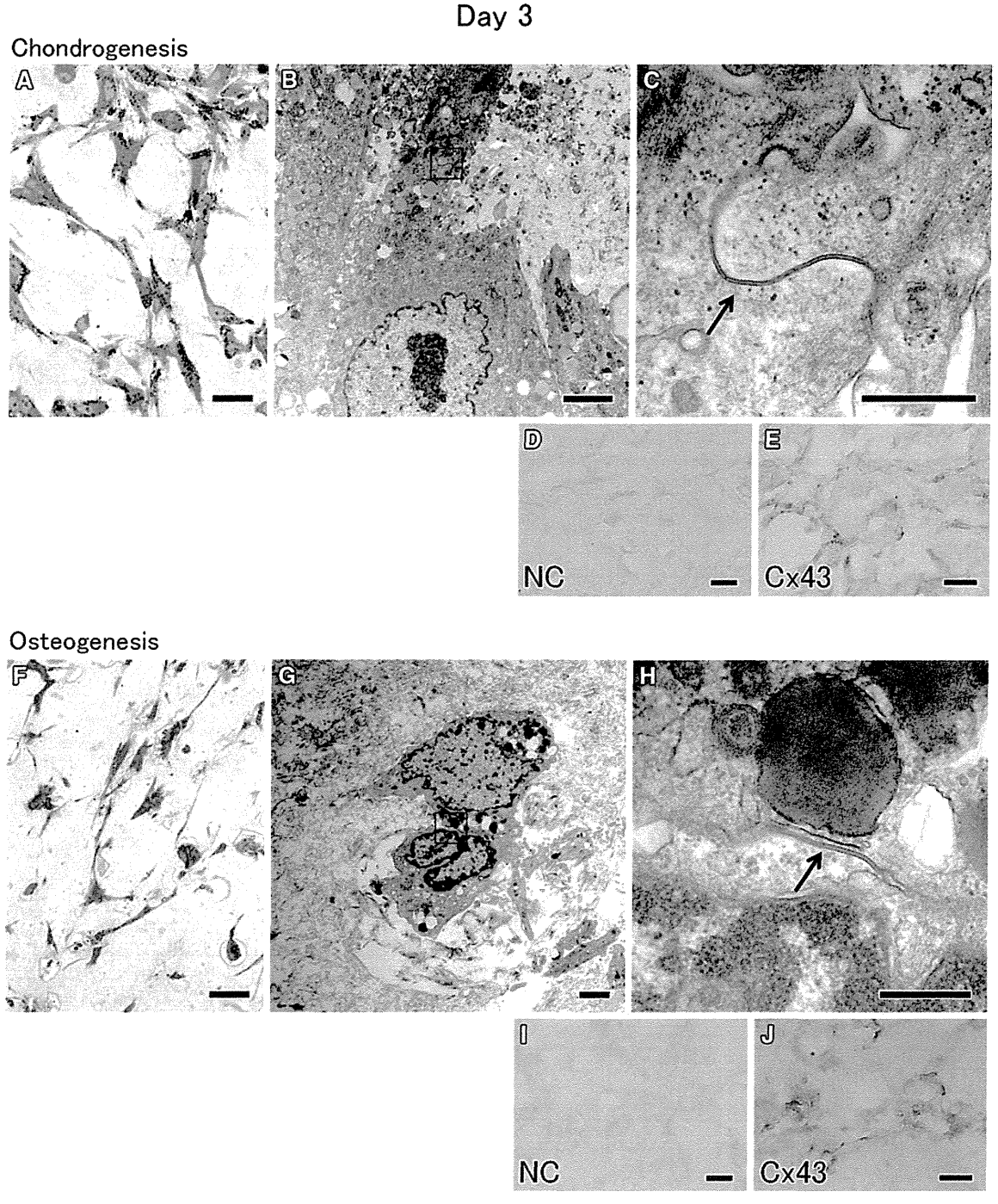

Fig. 3 MSC/collagen composite 3 days after chondrogenic (**a–e**) and osteogenic (**f–j**) induction. **a, f** Light micrographs of the composite stained with toluidine blue. **b, g** TEM images of MSC. **c, h** Higher magnifications of the areas indicated by the *squares* in **b** and **g**, respectively. The junctional complexes consisting of gap junctions are indicated by *arrows*. **d, e, i, j** Immunohistochemistry of negative control (*NC*) (**d, i**) and connexin 43 (*Cx43*) (**e, j**). *Bars* **a, d–f, i, j** 20 μm; **b, g** 5 μm; **c, h** 500 nm

Early phase of differentiation

At day 7, remarkable morphological differences were first observed during chondrogenesis and osteogenesis. During chondrogenesis, the shape of MSCs further changed from elongated to polygonal, and their cell processes decreased in number (Fig. 4a, b). Collagen type I level decreased, collagen type II was first detected, and a high level of chondroitin sulfate-proteoglycan was detected (Fig. 4c–f). During osteogenesis, MSCs remained spindle shaped with long cell processes (Fig. 4g, h). Osteocalcin and osteopontin, in addition to collagen type I, were detected extracellularly (Fig. 4i–l).

Late phase of differentiation

During chondrogenesis, the extracellular matrix around MSCs was stained purple (Fig. 5a). The cells had irregular outlines, well-developed rough endoplasmic reticulum, and Golgi apparatus. In addition, the cells were surrounded by lacunae (Fig. 5b). Collagen type I was maintained at a moderate level, whereas collagen type II and chondroitin sulfate-proteoglycan levels further increased (Fig. 5c–f).

During osteogenesis, dark blue particles were prominently seen around the spindle-shaped cells (Fig. 5g). Electron-dense needle-like crystals (Fig. 5h) were identified as hydroxyapatite (HAp) according to their electron

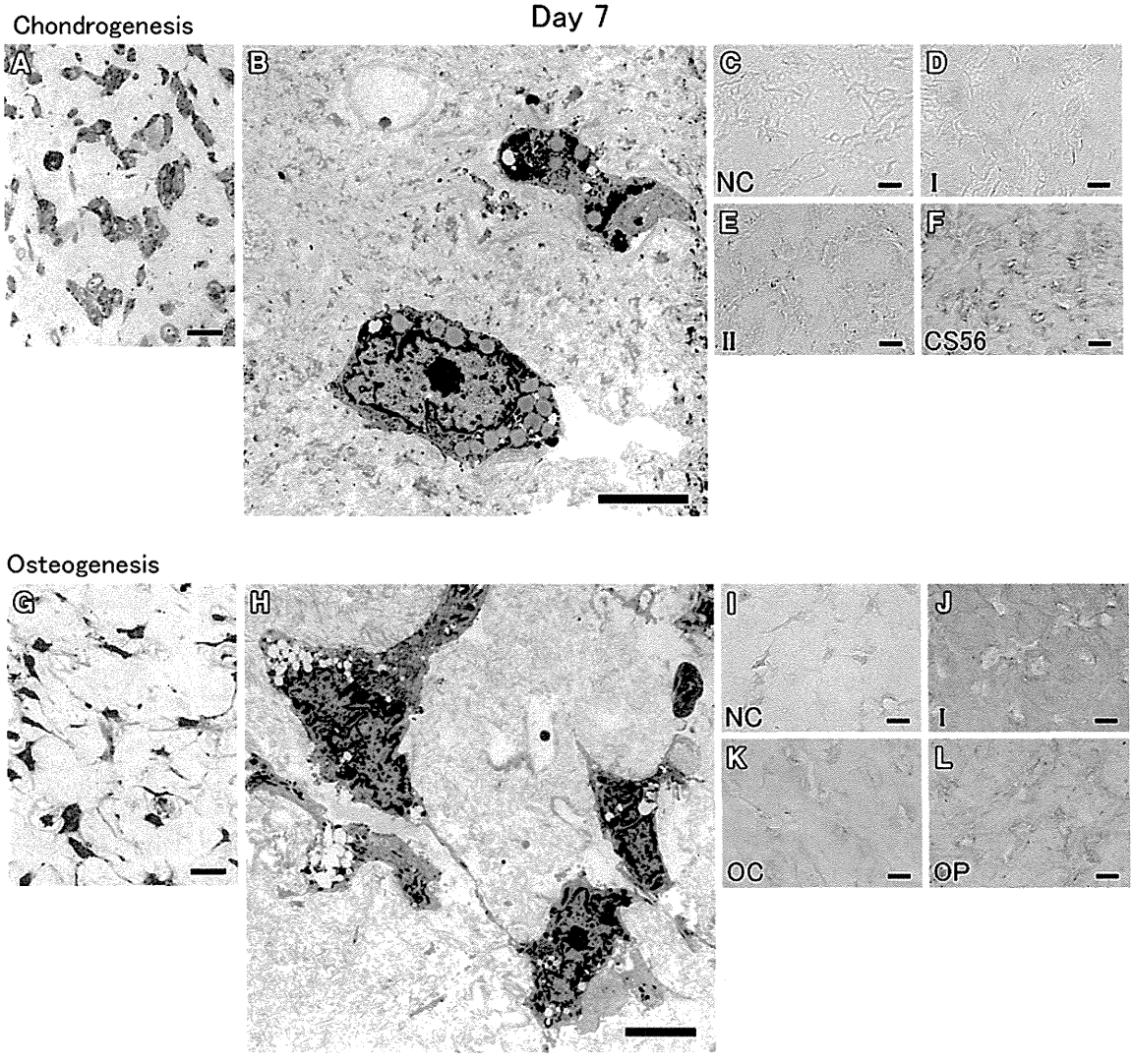

Fig. 4 MSC/collagen composite 7 days after chondrogenic (**a–f**) and osteogenic (**g–l**) induction. **a**, **g** Light micrographs of the composite stained with toluidine blue. **b**, **h** TEM images of MSC. **c–f**, **i–l** Immunohistochemistry of negative control (*NC*) (**c**, **i**), collagen type *I* (**d**, **j**), collagen type *II* (**e**), chondroitin sulfate-proteoglycan (*CS56*) (**f**), osteocalcin (*OC*) (**k**), and osteopontin (*OP*) (**l**). *Bars* **a**, **c–g**, **i–l** 20 μm; **b**, **h** 5 μm

beam diffraction pattern (Fig. 5h). The cells contained mitochondria, endoplasmic reticulum, and Golgi apparatus lying within a bone lacuna, and they were connected by gap junctions (Fig. 5i, j). Collagen type I level was maintained, whereas osteocalcin and osteopontin levels further increased (Fig. 5k–n).

The HAp crystals were further analyzed using energy-dispersive X-ray spectroscopy (EDS). SEM–EDS demonstrated that the HAp crystals were composed of calcium and phosphorus, which matched the characteristics of HAp (Fig. 6a). Collagen type I was observed around the HAp crystals by immunoelectron microscopy (Fig. 6b).

Discussion

For cartilage formation, the aggregation of chondroprogenitors is the first step [20, 21]. We previously examined morphological events during chondrogenesis of MSCs in a pellet culture system [22, 23] in which MSCs were pelleted without scaffold and cultured in chondrogenic medium. In this study, MSCs were embedded in collagen gel and cultured in similar chondrogenic medium. The events of the aggregation phase, the early phase of differentiation, and cartilage formation were commonly seen in both systems, the pellet culture system and the collagen gel culture system.

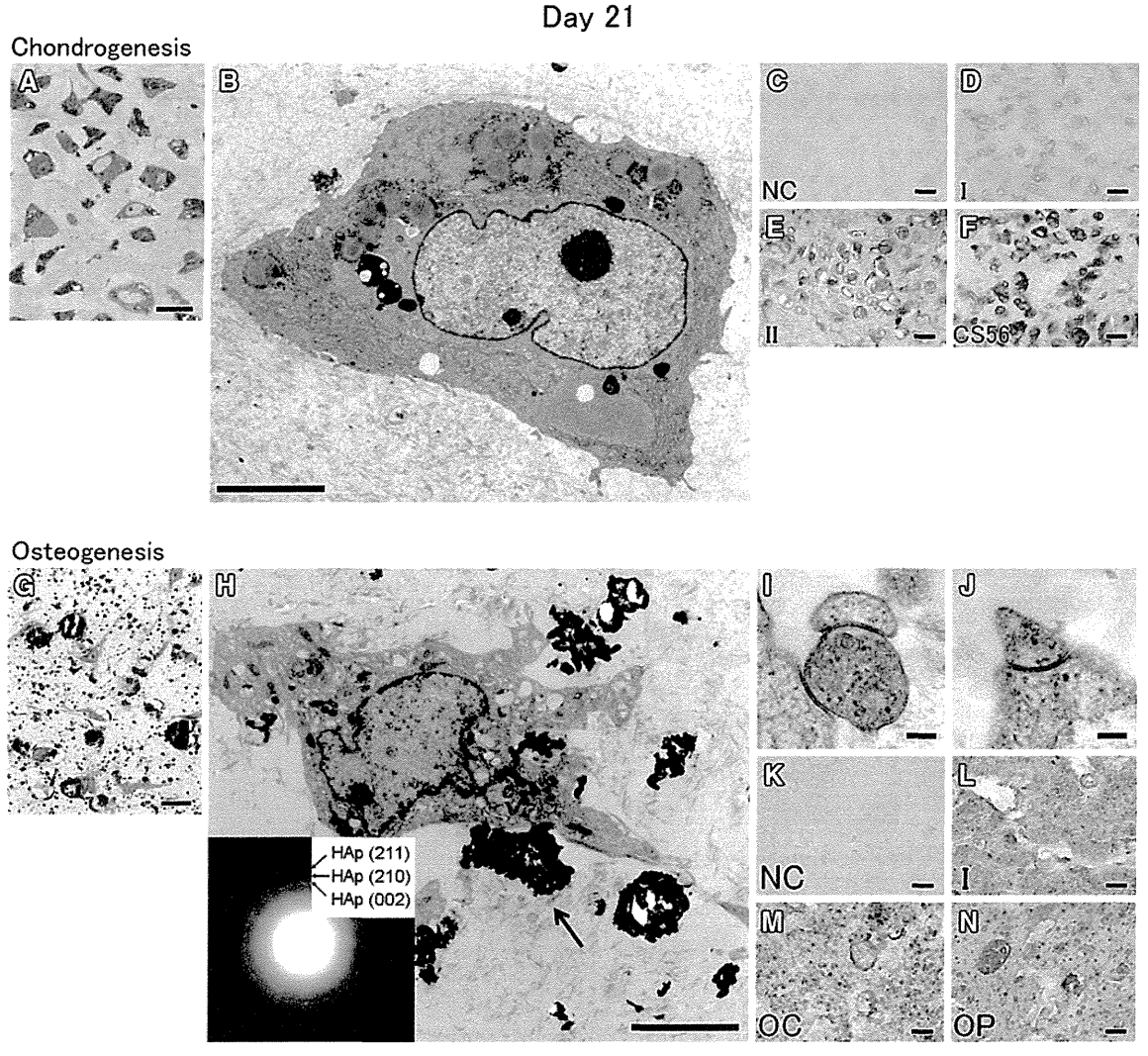

Fig. 5 MSC/collagen composite 21 days after chondrogenic (**a–f**) and osteogenic (**g–n**) induction. **a**, **g** Light micrographs of the composite stained with toluidine blue. **b**, **h** TEM images of MSC. Electron-dense particles are indicated by *arrows* in **h**, and their electron beam diffraction pattern is shown in the *square* (*HAp* hydroxyapatite) in **h**. **i**, **j** TEM images of gap junctions. **c–f**, **k–n** Immunohistochemistry of negative control (**c**, **k**), collagen type I (**d**, **l**), collagen type II (**e**), chondroitin sulfate-proteoglycan (**f**), osteocalcin (**m**), and osteopontin (**n**). *Bars* **a**, **c–g**, **k–n** 20 μm; **b**, **h** 5 μm; **i**, **j** 100 nm

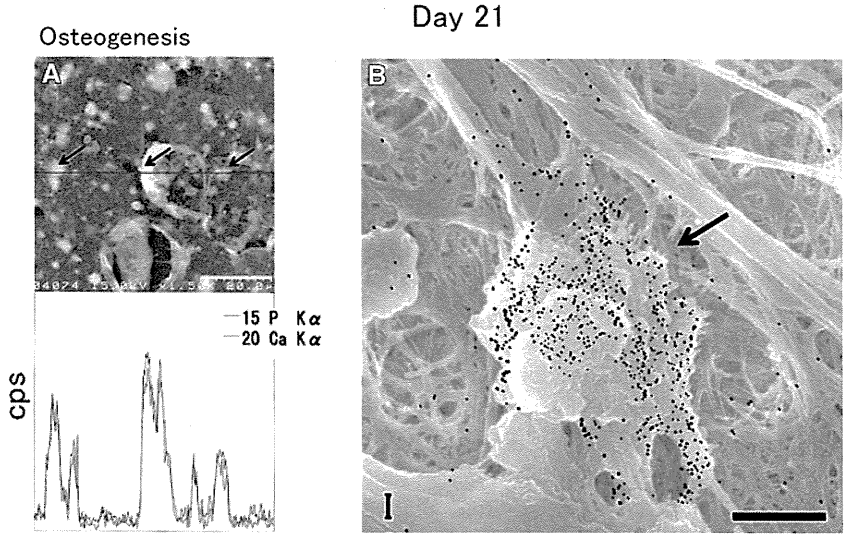

Fig. 6 X-ray and immunoelectron microscopic analysis for particles observed 21 days after osteogenic induction. **a** Line analysis using energy-dispersive X-ray spectroscopy for the particles (*arrows*). **b** Immunoelectron microscopic image by SEM for collagen type *I*. Particle is indicated by *arrow*. *Bars* **a** 20 μm, **b** 500 nm

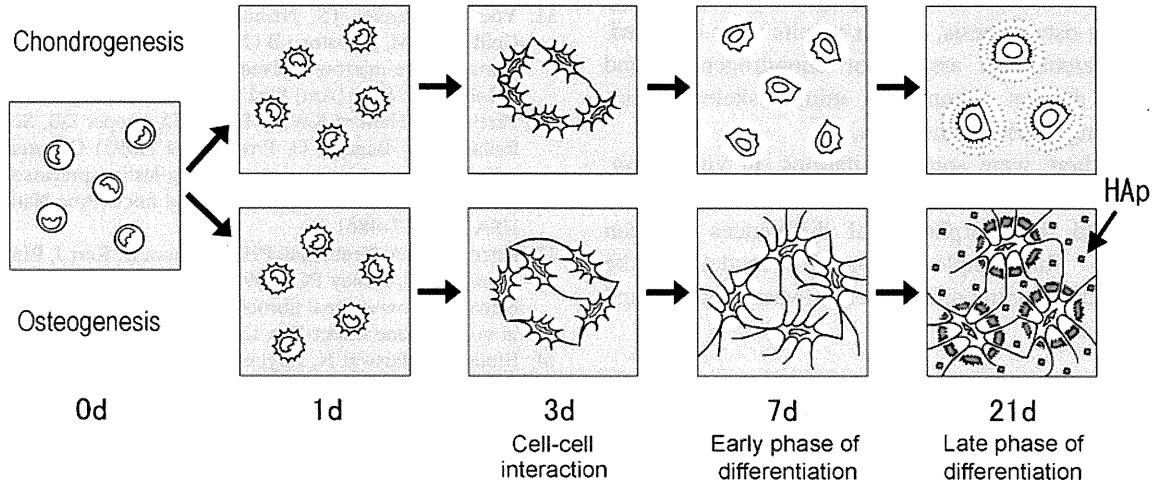

Fig. 7 Summary for comparative morphological analyses of MSCs during in vitro chondrogenesis and osteogenesis. Before inductions, MSCs were round in shape and dissociated in the collagen gel. At day 1, the shape of some MSCs was changed. At day 3, MSCs became elongated with long processes, and gap junctions were observed. At day 7, during chondrogenesis, the shape of MSCs further changed from elongated to polygonal. During osteogenesis, MSCs remained spindle shaped with long cell processes. At day 21, MSCs were surrounded with cartilage and bone matrix. *HAp* hydroxyapatite

Bone formation is a complex process involving the recruitment of osteoprogenitor cells to the bone surface and differentiation into mature osteoblasts that mineralize the extracellular matrix. MSC/collagen composites were reported to be an in vitro osteogenesis model by other groups [24, 25]. However, detailed morphological profiles and the microenvironment that occur during osteogenesis are unknown. In this study, we revealed that the MSCs were surrounded by an extracellular matrix composed of collagen type I, osteopontin, osteocalcin, and HAp particles. These findings support the MSC/collagen composite as one of the simplest models for bone formation.

At day 3, gap junctions were observed. Stains et al. [26] reported that osteoblasts achieved interactions through cadherin-based adhering junctions as well as gap junctions. On the other hand, gap junctional communication is necessary for the development and maintenance of a differentiated osteoblast phenotype [27–30]. In our previous pellet culture system for chondrogenesis, junctional complexes, desmosomes, and intermediate junctions were already observed at day 1 [31]. In this study, gap junctions were observed at day 3. These differences should have been caused by the initial cell density [31].

At day 7, remarkable morphological differences were first observed during chondrogenesis and osteogenesis. The shape of MSCs changed from elongated to polygonal without cell processes during chondrogenesis, whereas MSCs remained spindle shaped with long processes during osteogenesis. Concurrently, phenotypic markers, which are chondroitin sulfate-proteoglycan for chondrogenesis and

osteopontin and osteocalcin for osteogenesis, were first detected. These findings indicate that critical morphological changes to distinguish chondrogenesis and osteogenesis occurred between day 4 and day 6 in this model.

At day 21, extracellular matrix around MSCs was stained with collagen type II and chondroitin sulfate-proteoglycan during chondrogenesis, and the existence of hydroxyapatite and staining of collagen type I, osteopontin, and osteocalcin were confirmed extracellularly during osteogenesis. These findings demonstrated that MSC/collagen composite differentiated into cartilage and bone-like tissues in vitro.

In this study, MSCs cultured in specific conditions for osteogenesis and chondrogenesis accompanied lipid droplets, specific features for adipogenesis. We previously investigated a gene profile of MSCs during in vitro chondrogenesis of MSCs and reported that several specific markers for chondrogenesis dramatically increased [22], but some specific markers for adipogenesis also slightly increased. For osteogenesis, similar results were obtained. In vitro differentiation assays for chondrogenesis and osteogenesis do not completely mimic skeletogenesis observed during in vivo conditions.

Although there were some limitations, in vitro chondrogenesis and osteogenesis using an MSC/collagen composite clarified the morphological differences between them (Fig. 7). This simple comparative model will be helpful for understanding differences between cartilage and bone formation.

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Conflict of interest No competing financial interests exist.

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Human YKL39 (chitinase 3-like protein 2), an osteoarthritis-associated gene, enhances proliferation and type II collagen expression in ATDC5 cells

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ABSTRACT

Human YKL39 (chitinase 3-like protein 2/CHI3L2) is a secreted 39 kDa protein produced by articular chondrocytes and synoviocytes. Recent studies showed that hYKL-39 expression is increased in osteoarthritic articular chondrocytes suggesting the involvement of hYKL-39 in the progression of osteoarthritis (OA). However little is known regarding the molecular function of hYKL-39 in joint homeostasis. Sequence analyses indicated that hYKL-39 has significant identity with the human chitotorisidase family molecules, although it is considered that hYKL-39 has no enzymatic activity since it lacks putative chitinase catalytic motif. In this study, to examine the molecular function of hYKL-39 in chondrocytes, we overexpressed hYKL-39 in ATDC5 cells. Here we report that hYKL-39 enhances colony forming activity, cell proliferation, and type II collagen expression in these cells. These data suggest that hYKL-39 is a novel growth and differentiation factor involved in cartilage homeostasis.

1. Introduction

Osteoarthritis (OA) is a group of diseases and mechanical abnormalities involving degradation of articular cartilage and the subchondral bone. It is reported that OA affects 27 million people in the US (2005) and it is estimated that 80% of the US population will have radiographic evidence of OA by age 65 [1]. These statics indicate that the inhibition of cartilage loss and the promotion of cartilage repair are important issues to address, provided increases in life expectancy. Since OA is a multifactorial disease with factors such as aging, obesity, and joint instability, understanding the molecular pathophysiology of OA is important to develop more effective treatments. In this regard, many researchers have tried to identify the genes with altered expression patterns in OA cartilage [2].

hYKL-39 was discovered as a novel 39 kDa protein from the conditioned medium of human articular cartilage chondrocytes in primary culture [3]. It is currently recognized as a biochemical marker for the progression of osteoarthritis in humans [4]. However, the biological significance of hYKL-39 in cartilage homeostasis has not been appropriately defined.

hYKL-39 is closely related to other human proteins such as hYKL-40 (CHI3L1 [5]), chitotriosidase [6], AMCase (acid mammalian chitinase) [7], Oviductin [8], and SI-CLP (stabilin-1 interacting chitinase-like protein) [9]. All 6 proteins share significant sequence identity with bacterial chitinases and belong to the Glyco_18 domain-containing protein family [10]. Among these, only chitotriosidase and AMCase contain the putative chtinase catalytic motif, FDGxDxDxE, and are reported to have chitin hydrolytic activities. Since chitin is a major component of a diverse range of organisms such as insects, fungus, bacteria, nematodes, crustacean, and plants, it is considered that these two proteins are involved in the innate immune system [7,11]. In contrast, the other 4 proteins including hYKL-39 are reported to have no chitin hydrolytic activity and are classified as chitinase-like proteins (CLPs) or chi-lectins. Oviductin is exclusively expressed and secreted by oviductal epithelium and was reported to enhance in vitro fertilization rates in humans [12]. However, the specific function of Oviductin in this process remains to be established since no abnormality in the fertilization process was observed in mice null for Oviductin. SI-CLP was reported to be a binding protein for stabilin-1, a scavenger receptor expressed by macrophages and is considered to be involved in host defense and inflammatory reactions. Both hYKL-39 and hYKL-40 are reported to be expressed in joint tissues [4]. hYKL-40 is reported to be involved in the process of cell proliferation and extracellular matrix protein expression in articular chondrocytes [13]. Similar to hYKL-40, recent studies reported that

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