3 日間培養したが、より長期間分化誘導をかけた iPSCs を用いれば、*in vivo* での更なる軟骨分化誘導が期待できると考えられる。

本研究では、移植直前に予め軟骨分化誘導培地での培養をした後に欠損部への移植を行ったため、細胞が分化もしくは形質転換し、腫瘍が形成されなかった可能性も考えられる。過去の報告で、MSCs が産生される TGF-β が免疫寛容に寄与していることが報告されているが、軟骨欠損モデルラットへのマウス MSCs およびマウス iPSCs の移植が可能だった理由として、移植細胞が産生する TGF-β が関与しているかもしれない。

今後は、SCID マウスを用いて免疫拒絶の影響を最低限にし、軟骨再生能をより長期に解析することが期待される。患者自己 MSCs による軟骨再生治療はすでに臨床で行われているのに対し、iPSCs による軟骨再生は未だに基礎研究の段階である。本研究により、より安全な軟骨移植治療の実現化に向けた技術開発が発展することを期待する。

E. 結論

本研究では、軟骨欠損モデルラットにマウスの MSCs および iPSCs を移植し、軟骨細胞様の分化を確認することに成功した。*in vitro* と *in vivo* での軟骨分化誘導能力には相関があり、この知見を利用した安全な軟骨再生治療の実現が可能になると考えられる。

F．健康危険情報

報告すべき健康被害、健康危険情報はない。

G. 研究発表

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マウス間葉系幹細胞を用いた軟骨再生治療の有効性の検討
2014.3 第 13 回日本再生医療学会総会
京都

馬渕洋、緒方勇亮、鈴木喜晴、松崎有未、宗田　大、関矢一郎、赤澤智宏：
組織間葉系幹細胞の分化指向性の解析
2014.3 第 13 回日本再生医療学会総会
京都

H．知的財産権の出願・登録状況

1.特許取得
該当無し

2.実用新案登録
該当無し

3.その他
該当無し

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Atesok K, Doral MN, Bilge O, **Sekiya I.**	Synovial stem cells in musculoskeletal regeneration.	J Am Acad Orthop Surg.	21(4):	258-9. doi: 10.5435/J AAOS-21-04-258.	2013 Apr;
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関矢一郎	滑膜幹細胞による軟骨再生医療の開発	今日の移植	27(1)	53-60	2014
中村智祐 関矢一郎 宗田 大 小林英司	滑膜間葉系幹細胞による軟骨再生治療：ミニブタモデルでの検討	CLINICAL CALCIUM	32(12)	49-57	2013

研究成果の刊行に関する一覧表

書籍

発表者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
関矢一郎	関節軟骨損傷	福林　徹（監修）篠塚昌述（編集）	スポーツ整形外科マニュアル	中外医学社	東京	2013	p194-196
関矢一郎	変形性膝関節症	福林　徹（監修）篠塚昌述（編集	スポーツ整形外科マニュアル	中外医学社	東京	2013	p197-200
宗田　大	ひざ痛を治す	宗田　大（総監修）	別冊 NHK きょうの健康	NHK きょうの健康	東京	2013	P4~
関矢一郎	手術でひざの痛みを改善する	宗田　大（総監修）	別冊 NHK きょうの健康	NHK きょうの健康	東京	2013	p66-79
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北條浩彦、清水則夫	基本編－原理と基本知識－ リアルタイム PCR を使った解析の基本 10 プライマー／プローブの設計手順②マルチプレックスの場合	北條浩彦	原理からよくわかるリアルタイム PCR 完全実験ガイド 最強のステップ UP シリーズ	株式会社羊土社	東京	2013	p72-74

清水則夫、渡邊健、外丸靖浩	実践編－プロトコールを中心に－ Ⅳ章 遺伝子量解析 15 ウイルス感染症を診断するウイルスゲノムの定性的検査と定量的検査	北條浩彦	原理からよくわかるリアルタイムPCR 完全実験ガイド 最強のステップ UP シリーズ	株式会社羊土社	東京	2013	p192-202

Ⅳ．研究成果の刊行物・別刷

Synovial Stem Cells in Musculoskeletal Regeneration

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Topics from the frontiers of basic research presented by the Orthopaedic Research Society.

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Dr. Atesok or an immediate family member serves as a board member, owner, officer, or committee member of the International Society of Arthroscopy, Knee Surgery, and Orthopaedic Sports Medicine and the Orthopaedic Research Society. Dr. Doral or an immediate family member serves as a board member, owner, officer, or committee member of the International Society of Arthroscopy, Knee Surgery, and Orthopaedic Sports Medicine. Neither of the following authors nor any immediate family member has received anything of value from or has stock or stock options held in a commercial company or institution related directly or indirectly to the subject of this article: Dr. Bilge and Dr. Sekiya.

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The synovium is a thin layer of connective tissue that lines the joint surface, tendon sheaths, and bursae at freely moving articulations in the body. Embryologic origin of the synovial tissue is the mesenchymal layer, which also gives rise to bone, cartilage, ligament, and muscle tissue. The synovium has several functions, including lubrication of the articulating surfaces, nutrition of articular cartilage, and regulation of immune response within the joint.

Synovial Cells

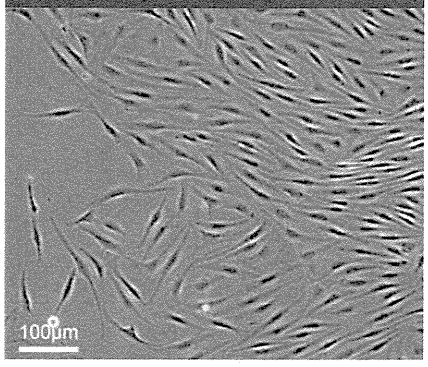
The synovium contains two main cell types: type A and type B synoviocytes. Type A synoviocytes are tissue macrophages and have phagocytic functions. Type B synoviocytes are fibroblast-like cells and function in the formation of synovial fluid.[1] In 2001, De Bari et al[2] isolated mesenchymal stem cells (MSCs) from the synovium (Figure 1). Type A synoviocytes can be characterized and eliminated from mixed synovial cell populations through a selective culturing process. However, type B synoviocytes and synovial-MSCs have similar phenotypic features, and specific characteristics to clearly differentiate these two cell types from each other have not been determined yet.[1]

In terms of immune phenotype, there are many similarities between synovial-MSCs and MSCs of other origins. Both cell types are positive for surface markers such as CD44, CD90, and CD105. However, cells derived from synovium, including synovial-MSCs, have higher expression of CD44 (a hyaluronan receptor) and can express uridine diphosphoglucose dehydrogenase, which is a vital enzyme involved in hyaluronan synthesis.[3] In vitro studies have shown that synovial-MSCs have superior potential to differentiate into chondrocytes and to produce cartilage compared with MSCs of other origins.[4] Moreover, synovial-MSCs have greater proliferation and colony-forming capacity than do other stem cell sources.[4]

Synovial-MSCs and Cartilage Regeneration

Based on the promising results from in vitro studies, investigators have launched animal model studies to evaluate the effects of synovial-MSCs in vivo. In a rabbit model with full-thickness articular cartilage defect, Koga et al[5] demonstrated that local transplantation of synovial-MSCs results in extensive cartilage matrix formation at the defect site. These authors also observed that in the deeper zone of the defect, synovial-MSCs differentiated into bone cells, whereas synovial-MSCs at the superficial zones differentiated into chondrocytes. This observation supported the multilineage differentiation potential of synovial-MSCs according to local microenvironments in vivo. In a pig model, transplantation of synovial-MSCs into a full-thickness articular cartilage defect promoted cartilage regeneration based on arthroscopic, MRI, and histologic analysis as early as 3 months after the procedure.[6] Bilge et al[7] used a rabbit knee model as an in vivo culture medium to evaluate the effects of synovium on chondrocyte growth. These authors observed that cartilage grafts

Figure 1



Histologic appearance of human synovial mesenchymal stem cells under the phase contrast microscope 14 days after initial plating.

that are in direct contact with the synovium produce more chondrocytes compared with cartilage grafts that are not. Studies of animal meniscal defect models demonstrated that transplanted synovial-MSCs adhere to sites of meniscal injury, differentiate into cells resembling meniscal fibrochondrocytes, and enhance meniscal regeneration.[8,9]

Sporadic human studies have reported that the number of synovial-MSCs in synovial fluid increases in knees with degenerated cartilage and osteoarthritis and following intra-articular ligament injury.[10,11] This observation raises the question whether the number of synovial-MSCs that are mobilized from synovium into synovial fluid increases according to the degree of cartilage degeneration as part of the reparative process. Human trials investigating the effects of intra-articular synovial-MSC transplantation to promote cartilage regeneration and/or to prevent osteoarthritis should answer this question.

Application of Synovial-MSCs for Bone, Tendon, and Muscle Regeneration

Synovial-MSCs may offer an alternative cell-based treatment strategy for bone, tendon, and muscle regeneration. In a rabbit bone defect model, Matsusaki et al[12] demonstrated that using tissue-engineered construct derived from synovial-MSCs with hydroxyapatite accelerates osteoinduction. In a rat Achilles tendon graft model, synovial-MSC implantation into bone tunnel accelerated early remodeling of tendon-to-bone healing.[13] Another rat model study showed that synovial-MSCs have myogenic potential and contribute to skeletal muscle regeneration in vivo.[14] In spite of these reports, there is not sufficient evidence regarding osteogenic and myogenic potential of synovial-MSCs compared with bone marrow– and muscle-derived MSCs.

Future Perspectives

In vitro and animal model studies support the use of synovial-MSCs in cartilage regeneration as an important, arguably superior, cell-based treatment alternative. Further investigations in the near future should help in our understanding the complexity of synovial-MSC biology in terms of isolation, characterization, culturing, distinguishing from, and interacting with other cell types before this promising cell-based therapy can be translated into clinical practice.

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Med Mol Morphol (2013) 46:24–33
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ORIGINAL PAPER

Comparative sequential morphological analyses during in vitro chondrogenesis and osteogenesis of mesenchymal stem cells embedded in collagen gels

Shizuko Ichinose · Motoki Tagami · Takeshi Muneta · Hitoshi Mukohyama · Ichiro Sekiya

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Abstract Chondrogenesis and osteogenesis during fetal development and postnatal growth constitute one of the most interesting and complicated subjects in biology. In this study, bone marrow mesenchymal stem cells (MSCs) were embedded in collagen gel, cultured in chondrogenic or osteogenic medium, and compared morphologically during chondrogenic and osteogenic differentiation sequentially by light and electron microscopy and immunohistochemical examination. Before induction, the MSCs were dispersed and round in the collagen gel. At day 1, MSCs with a large number of short processes produced extracellular fibers whose immunoreactivity was positive for collagen type I. At day 3, the shape of MSCs changed from round to elongated. Gap junctions positive for connexin 43 were also observed. At day 7, remarkable morphological differences were first observed during chondrogenesis and osteogenesis. The shape of MSCs changed to polygonal without cell processes during chondrogenesis, while MSCs remained spindle shaped with long processes during osteogenesis. Concurrently, collagen type II during chondrogenesis and osteocalcin during osteogenesis were first detected. At day 21, chondrogenesis and osteogenesis of the MSC/collagen composite further progressed, respectively. In vitro chondrogenesis and osteogenesis using an MSC/collagen composite clarified the morphological differences.

Keywords Human mesenchymal stem cells · Mesenchymal stem cell–collagen composite · In vitro chondrogenesis and osteogenesis · Cell–cell interaction · Differentiation

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Introduction

Most bones develop through a process known as endochondral ossification [1]. Cartilage and bone formation during fetal development and postnatal growth is one of the most interesting and complicated subjects in biology. One speculation is that common progenitors contribute to both cartilage and bone development. However, the common progenitors cannot be distinguished from other cells making it difficult to perform morphological analyses of the common progenitors during both cartilage and bone formation. To address this problem, a simple experimental system is required.

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).

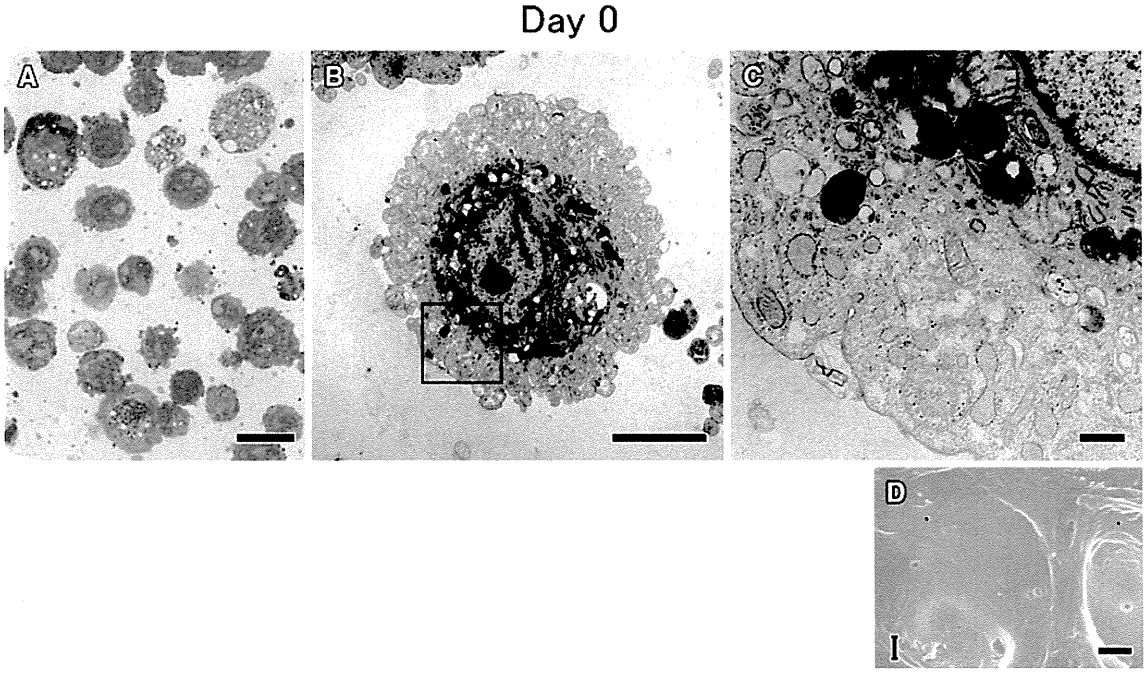

Fig. 1 Mesenchymal stem cell (MSC)/collagen composite before chondrogenic and osteogenic induction. **a** Light micrograph of composite stained with toluidine blue. **b** Transmission electron microscopy (TEM) image of MSC. **c** Higher magnification of the area indicated by the *square* in **b**. **d** 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).

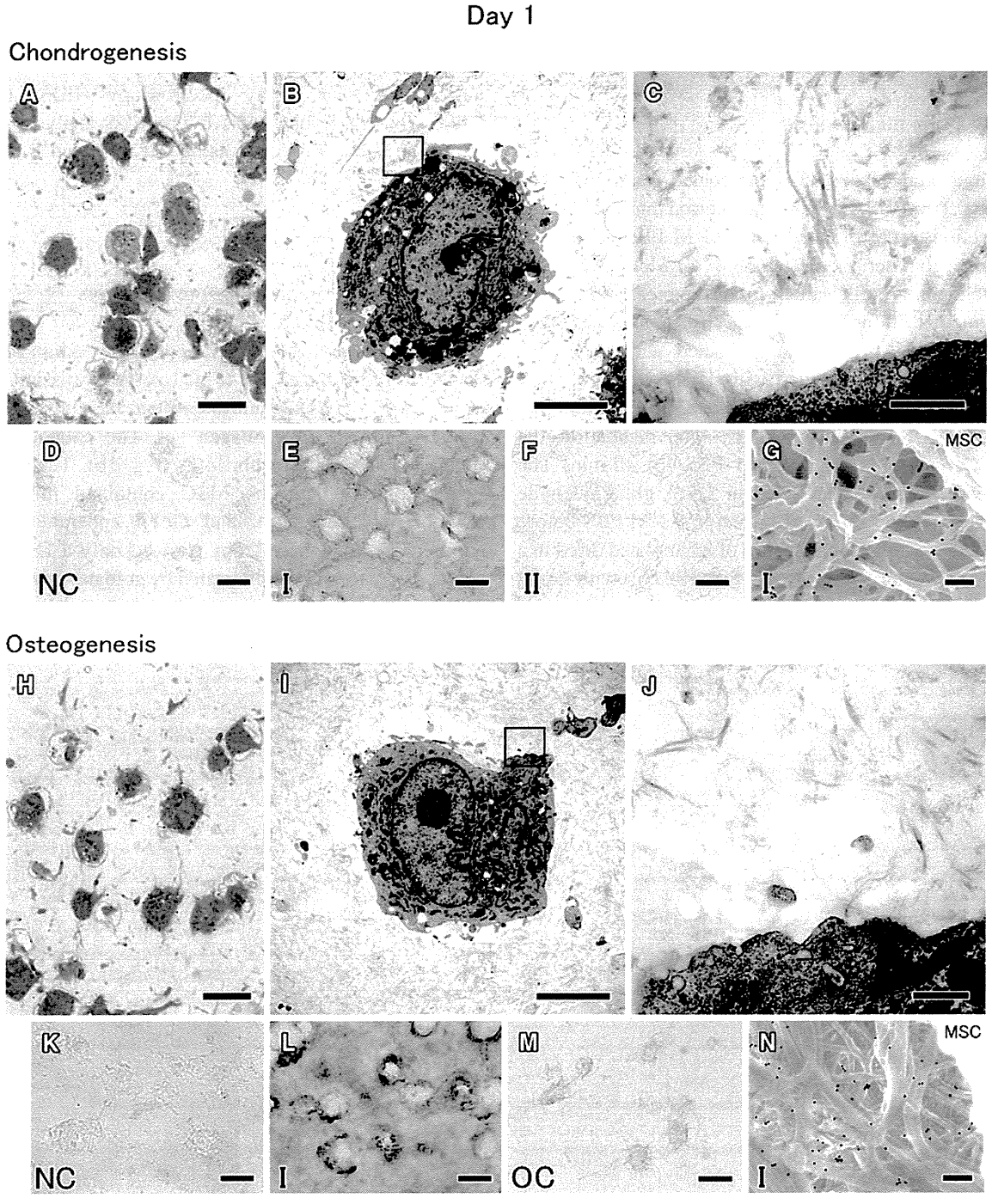

Fig. 2 MSC/collagen composite 1 day after chondrogenic (**a–g**) and osteogenic (**h–n**) induction. **a**, **h** Light micrographs of the composite stained with toluidine blue. **b**, **i** TEM images of MSC. **c**, **j** Higher magnifications of the areas indicated by the *squares* in **b** and **i**, 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.

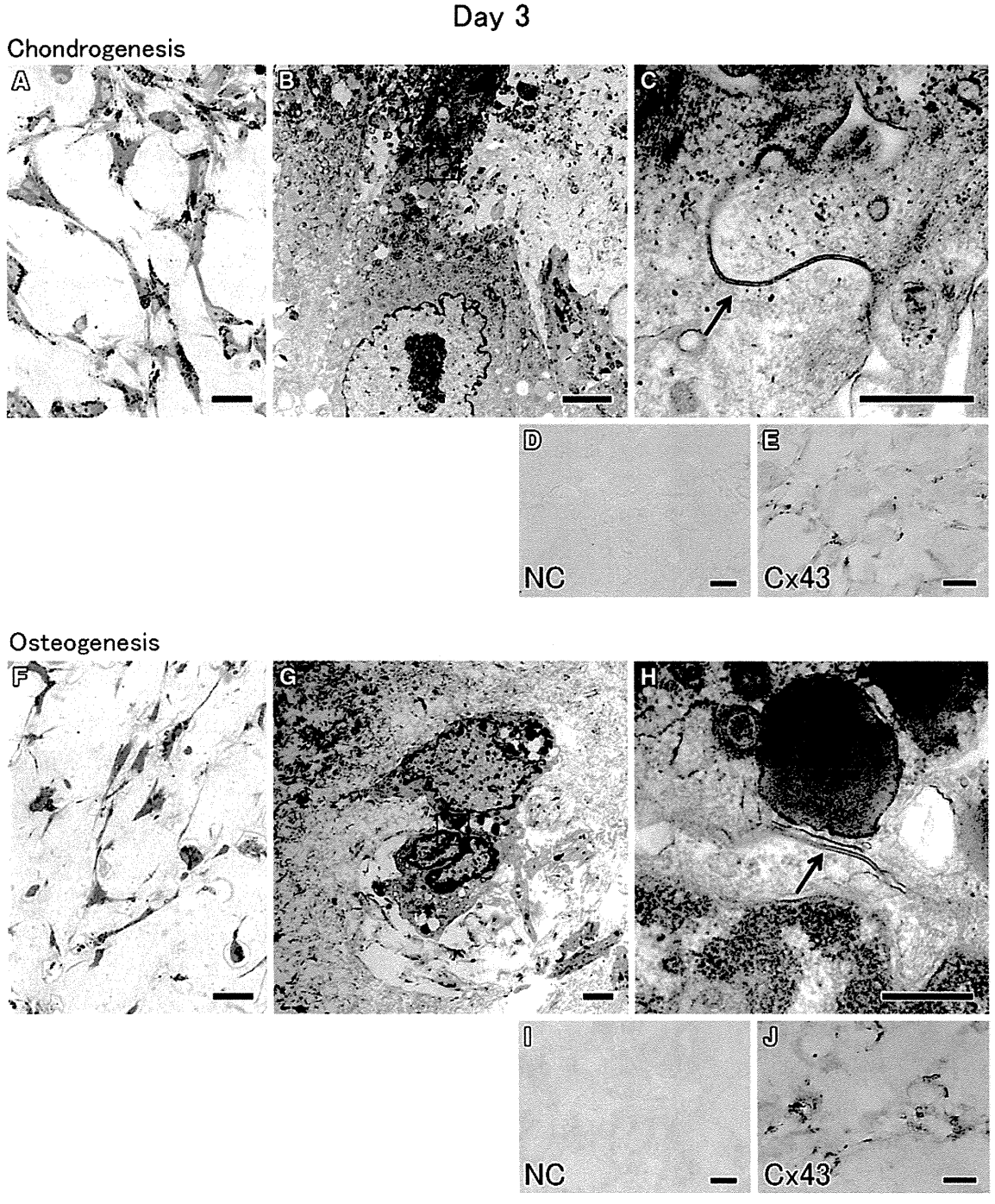

Fig. 3 MSC/collagen composite 3 days after chondrogenic (**a**–**e**) and osteogenic (**f**–**j**) induction. **a**, **f** Light micrographs of the composite stained with toluidine blue. **b**, **g** TEM images of MSC. **c**, **h** Higher magnifications of the areas indicated by the *squares* in **b** 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

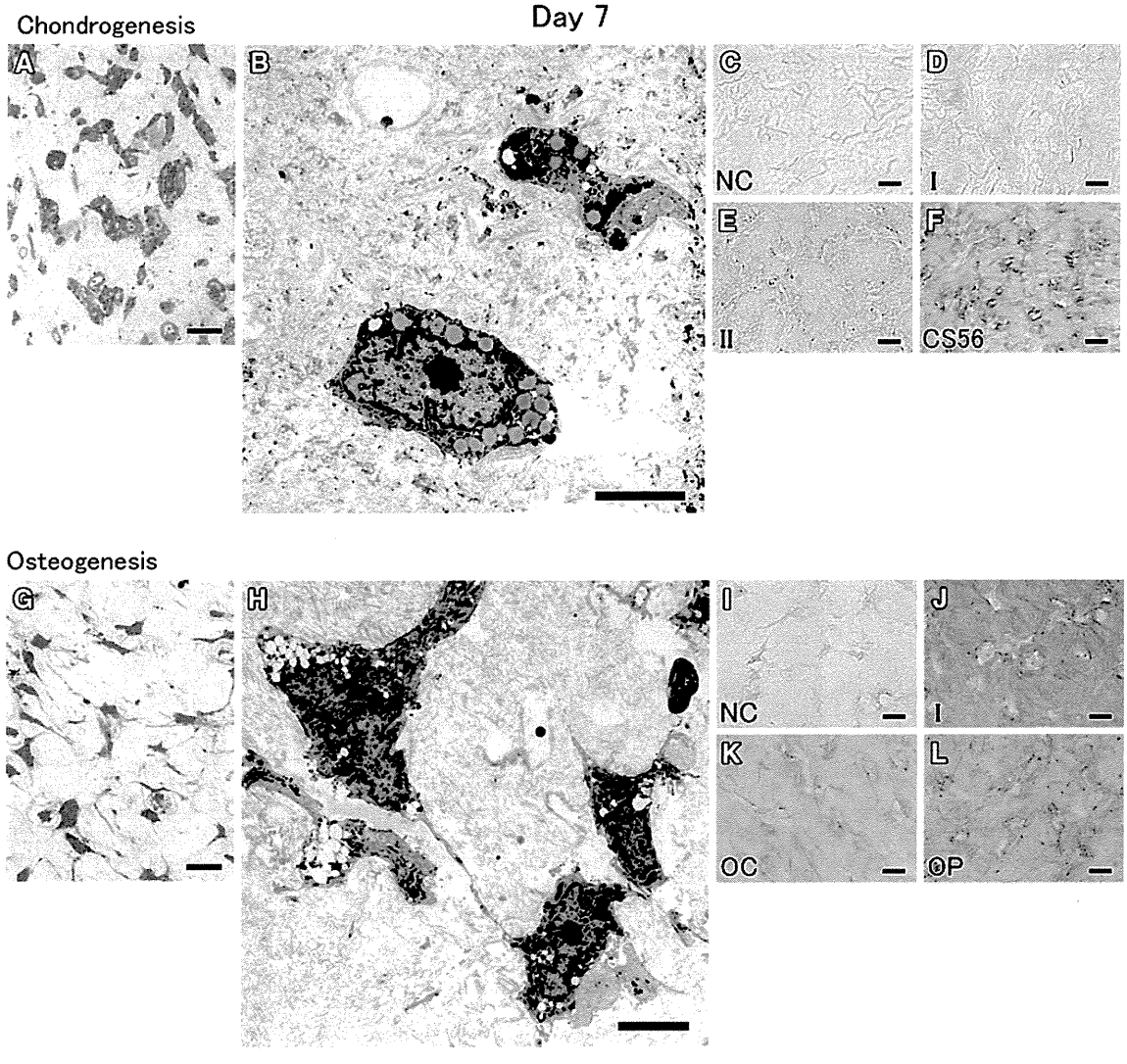

Fig. 4 MSC/collagen composite 7 days after chondrogenic (**a–f**) and osteogenic (**g–l**) induction. **a**, **g** Light micrographs of the composite stained with toluidine blue. **b**, **h** TEM images of MSC. **c–f**, **i–l** Immunohistochemistry of negative control (*NC*) (**c**, **i**), collagen 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.

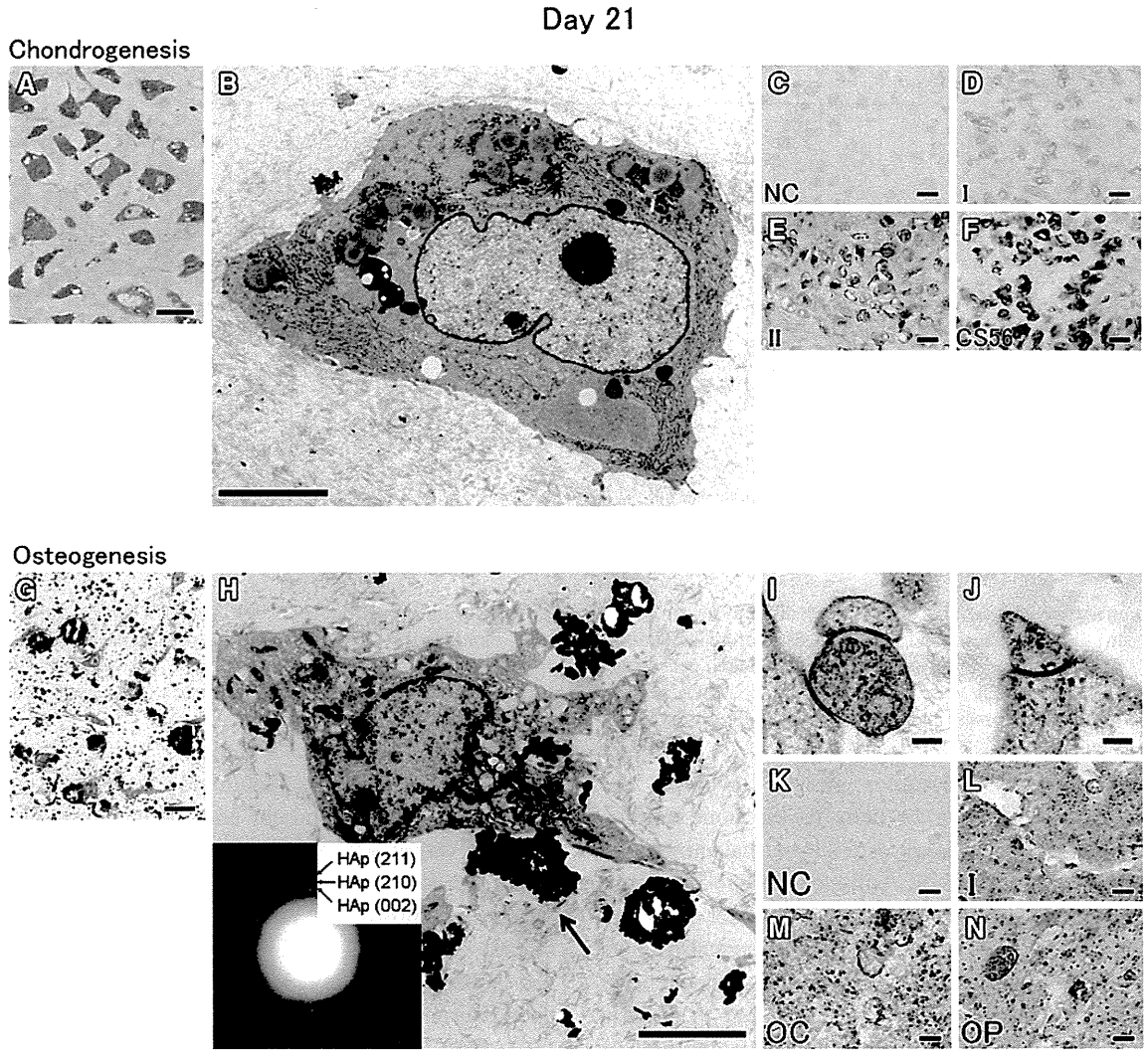

Fig. 5 MSC/collagen composite 21 days after chondrogenic (**a–f**) and osteogenic (**g–n**) induction. **a**, **g** Light micrographs of the composite stained with toluidine blue. **b**, **h** TEM images of MSC. Electron-dense particles are indicated by *arrows* in **h**, and their electron beam diffraction 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

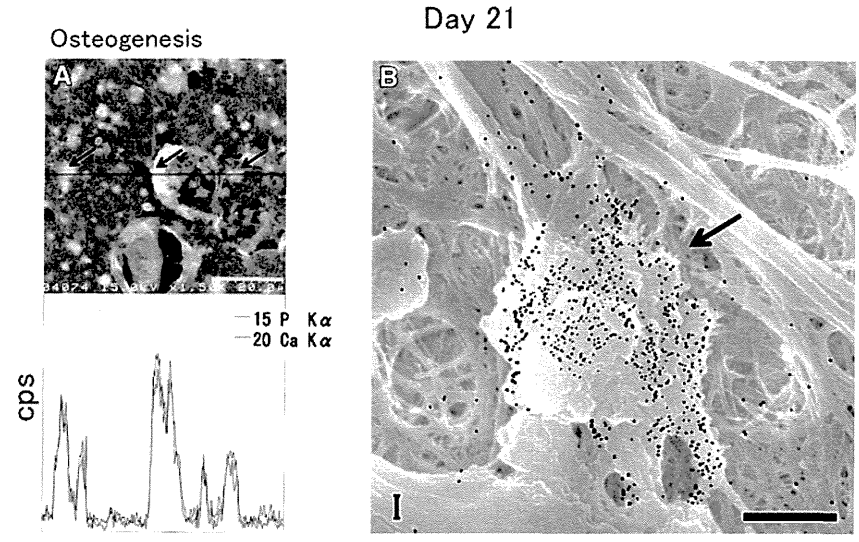

Fig. 6 X-ray and immunoelectron microscopic analysis for particles observed 21 days after osteogenic induction. **a** Line analysis using energy-dispersive X-ray spectroscopy for the particles (*arrows*). **b** Immunoelectron microscopic image by SEM for collagen type *I*. Particle is indicated by *arrow*.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

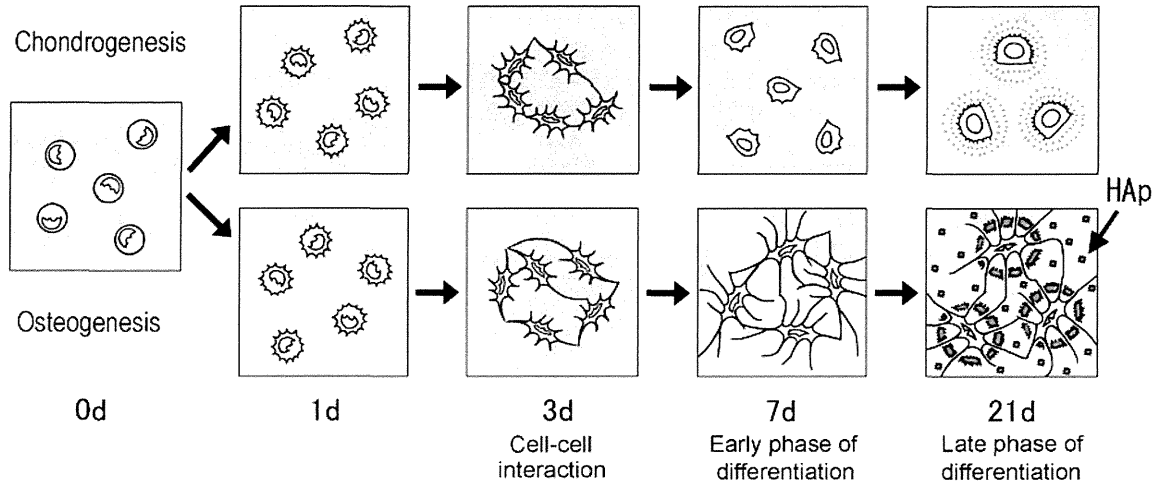

Fig. 7 Summary for comparative morphological analyses of MSCs during in vitro chondrogenesis and osteogenesis. Before inductions, MSCs were round in shape and dissociated in the collagen gel. At day 1, the shape of some MSCs was changed. At day 3, MSCs became elongated with long processes, and gap junctions 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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