Expression of osteogenic proteins during the intrasplenic transplantation of Meckel's chondrocytes: A histochemical and immunohistochemical study*

Kiyoto Ishizeki¹, Tadayoshi Kagiya¹, Naoki Fujiwara¹, Keishi Otsu¹², and Hidemitsu Harada¹

¹Department of Oral Anatomy II, School of Dentistry, and ²Adovanced Oral Health Science Research Center, Iwate Medical University, Morioka, Japan

Summary. Meckel's chondrocytes, derived from the ectomesenchyme, have the potential to transform into other phenotypes. In this study, we transplanted cell pellets of Meckel's chondrocytes into isogenic mouse spleens and analyzed their phenotypic transformation into osteogenic cells using histological and immunohistochemical methods. With the increasing duration of tranplantation, chondrocytes were incorporated into splenic tissues and formed a von Kossa-positive calcified matrix containing calcium and phosphoric acid, similar to that of intact bone. Type I, II, and X collagens, and the bone-marker proteins osteocalcin, osteopontin, osteonectin, and bone morphogenetic protein-2 (BMP-2) were immunolocalized in the matrix formed by the transplanted chondrocytes. Osteopontin and osteonectin were detected in the calcified matrix at earlier stages than osteocalcin and BMP-2. Type II collagen was expressed during the first week of transplantation, and type X collagen-positive cells appeared scattered during the initial stage of calcification, these collagens being later replaced by type I collagen formed by osteocyte-like cells. Electron microscopic observations revealed that chondrocytes surrounded by the calcified matrix transformed into spindle-shaped osteocytic cells accompanied the formation of bone-type thick-banded collagen fibrils. These results suggest that phenotypic switching of Meckel's chondrocytes can occur under in vivo conditions at a cellular morphological level.

Introduction

Skeletons of higher vertebrates are formed from either endochondral or intramembranous bone. In intramembranous ossification, bone tissue is formed directly by osteoblasts in the dense connective tissue without the involvement of chondrocytes. In endochondral ossification, however, mesenchymal cells first develop into a cartilaginous template through precartilaginous condensation, and the cartilage subsequently hypertrophies, is destroyed by vascular invasion, and is then replaced by trabecular bone and bone marrow (Weiss et al., 1986; Fang and Hall, 1997; Hall, 2005). It is generally accepted that chondrocytes undergo cellular hypertrophy, degenerate, and ultimately die at the osseous-cartilage junction during endochondral bone formation (Jee, 1988). However, it has been reported that some hypertrophic chondrocytes are released into marrow spaces in the growth plate cartilage of long
bones (Yoshioka and Yagi, 1980, 1988) and may thus be actively involved in bone formation (Holtrop, 1972; Silbermann and Frommer, 1972, 1974; Galotto et al., 1994) or acquired osteogenic potential by transformation into bone-forming cells including osteoblasts (Holtrop, 1972; Silbermann and Frommer, 1974; Galotto et al., 1994). Such phenotypic switching occurs more frequently in chondrocytes from the mandibular condylar cartilage than in those from long bone growth plates. Lewinson and Silbermann (1986), and Ben-Ami et al. (1993) reported that chondro-progenitor cells underwent osteogenic differentiation and formed new bone during a culture of mandibular condyles. Biochemical findings have suggested that, under certain culture conditions, not only chondrocytes in the progenitor zone of mandibular condyles, but also those in the maturation zone start to express genes typical of osteoblast differentiation (Celeste et al., 1986; Schmidt et al., 1986; Yoon et al., 1988; Lian et al., 1989). These two zones have also shown marked increases in mRNA expression for bone-type proteins such as alkaline phosphatase (ALPase), collagen type I, osteonectin, osteopontin, and osteocalcin during the organ culture of mandibular condyles isolated from newborn mice (Strauss et al., 1990). It is therefore noteworthy that chondrocytes from mandibular condyles can express genes characteristic of osteogenic differentiation. However, it is difficult to examine transforming cells at the cellular level in situ, and it is possible that osteogenic cells might be induced by contaminating perichondrial cells and/or immature chondrocytes.

We previously reported that chondrocytes from the midportion of Meckel's cartilage could undergo phenotypic transformation into ligament-forming fibroblastic cells in vivo (Harada and Ishizeki, 1998) and into osteocyte-like cells in vitro (Ishizeki et al., 1996a, b; Ishizeki et al., 1997; Ishizeki et al., 1998a, b), in response to local environmental conditions. In order to verify the origin-specific features of chondrocytes, we transplanted fragments of Meckel's cartilage and costal cartilage into a mouse spleen (Ishizeki et al., 1992a, b) and confirmed that Meckel's cartilage, which is derived from neural crest menenchymal cells, differed from mesoderm-derived costal cartilage; specifically, Meckel's chondrocytes transformed into osteogenic cells expressing bone-type proteins, while mesoderm-derived costal cartilage cells maintained chondrocytic features throughout the period of transplantation. These findings were confirmed under cell culture conditions (Ishizeki et al., 2003). Furthermore, a unique cartilaginous hard tissue called the chondroid bone, which includes both bone-specific type I collagen and cartilage-specific type II collagen in its matrix, is also known to derive from neural crest-originated ectomesenchymal cells (Goret-Nicaise and Dhem, 1982; Hall, 2005). We postulated that neural crest-derived cartilage has a common ability of osteogenic transformation, while mesoderm-derived cartilage undergoes a committed developmental process.

In order to test this hypothesis, we sought to determine whether intrasplenically transplanted Meckel's chondrocytes could recapitulate the process of osteocytic transformation. We also aimed to obtain data to support the idea that the cellular transformation of Meckel's chondrocytes reflected an intrinsic property of neural crest-derived chondrocytes.

**Materials and Methods**

**Animals**

Sixteen-day-old mouse embryos (vaginal plug = day 0) were dissected from pregnant mice (ddY strain) that had been killed with an overdose of CO2. The mandible—with the Meckel's cartilage—was removed from the embryonic mice under a microscope and placed in phosphate-buffered saline (PBS) containing 0.2% glucose (PBS-G) and kanamycin (60 μg/ml; Meiji Seika Co., Tokyo). After treatment with PBS containing 10 mM di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA) for 10 min, the Meckel's cartilage was extracted from the mandibular arch and transferred to fresh PBS-G. Special care was taken to remove the perichondrium surrounding the lateral side of Meckel's cartilage, because of the possibility that osteoprogenitor cells could be contained in this zone. All animal experiments were performed according to the Protocols for the Humane Treatment of Animals of Iwate Medical University.

**Cell source**

Chondrocytes for transplantation were isolated using 0.1% hyaluronidase (type I-S; Sigma Chemical Co., St. Louis, MO, USA) and 0.15% collagenase (type II; Worthington Biochemical Corp., Freehold, NJ, USA), as described previously (Ishizeki et al., 1996b, 1998b). Chondrocytes were then collected in a 10 ml centrifuge tube and washed three times with a-modified Eagle medium (α-MEM) containing 20% fetal bovine serum. The cells were washed again with PBS-G and centrifuged for 3 min at 1800 rpm. After gentle removal of the supernatant, cell pellets consisting of Meckel's chondrocytes were formed and used as chondrocyte aggregates for transplantation.
Expression of osteogenic proteins in chondrocytes

Transplantation
Recipient mice were anesthetized by an intraperitoneal injection of nembutal (0.6 mg/10 g body weight), as previously reported in detail (Ishizeki et al., 1987). Cell pellets were collected using a Venula-V-2 needle (Top Co. Ltd, Tokyo) and were transplanted into two sites in the spleen. Prior to extraction of the explants, the mice underwent vascular perfusion with 4% paraformaldehyde in PBS (pH 7.2).

Histological and histochemical observations

von Kossa staining
Explants transplanted for 1 and 4 weeks were stained using the von Kossa stain for the histochemical analysis of mineralization. Briefly, the specimens surrounded by splenic tissue were fixed with 4% paraformaldehyde, washed thoroughly in PBS, and incubated with 5% silver nitrate for 20 min for von Kossa staining (Ishizeki et al., 1996b).

Light and electron microscopy
For light microscopic observations, undecalcified semithin sections (1 μm) after 1 week of transplantation and decalcified semithin sections after 4 weeks of transplantation were prepared from samples embedded in epoxy-resin (Epon-812; Taab Laboratories Equipment Ltd, Aldermaston, UK) and stained with 0.1% toluidine blue (pH 3.0).

For electron microscopy, samples transplanted for 2 and 4 weeks were fixed in cold buffered 2.5% glutaraldehyde (pH 7.2) for 2–4 h and post-fixed in 1% osmium tetroxide, according to conventional procedures. After dehydration through a graded ethanol series, the specimens were embedded in Epon 812. Ultrathin sections without decalcification were cut with a diamond knife using an LKB-8800 ultratome (Bromma, Sweden). These sections were then stained with uranyl acetate and lead citrate prior to examination under an H-7100 (Hitachi, Tokyo) electron microscope.

Immunohistochemical procedures
Explants for immunostaining were harvested after 1, 2, and 4 weeks of transplantation and fixed in 4% paraformaldehyde for 30 min. The specimens at 4 weeks of transplantation were further decalcified for 2-5 days in PBS (pH 7.2) containing 4% EDTA. After washing with PBS, the explants were embedded in Tissue-Tek Compound 4583 (Sakura Finetechnical Co., Tokyo) prior to cutting with a cryostat. Cryosections at a 6-μm thickness were obtained from the frozen samples at -20°C, washed thoroughly with PBS, and then processed for examination after immunofluorescence and immunoperoxidase staining.

Types I, II, and X collagens were examined by immunoperoxidase staining to identify collagenous proteins in the explants. Slides were reactivated using a STUF MARK 2 unmasking solution kit (Serotec, Inc., Raleigh, NC, USA) for 10 min, and endogenous peroxidase was blocked by treatment with 3% H2O2 in methanol for 30 min at room temperature. After washing with PBS, specimens were incubated with rabbit anti-rat type I collagen antibody (LSL Co. Ltd., Tokyo) diluted 1:200 in PBS containing 0.1% bovine serum albumin, rabbit anti-rat type X collagen antibody (MAP, LSL) diluted 1:100 with PBS, or rabbit anti-bovine type II collagen (LSL) at a dilution of 1:200 for 1.5 h at 37°C. After washing, the specimens were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG second antibodies (CAPP-EL; Organon Teknika, Durham, NC, USA, 1:500 dilution) for 1 h at 37°C. Immunoreactivity was visualized with a diaminobenzidine (DAB) kit (Sigma) for 5–10 min, and then counterstained lightly with hematoxylin. Control cultures were incubated directly with the secondary antibodies in the absence of the primary antibodies and processed as above. No significant positive immunoreactivity was found in controls.

For immunofluorescence staining, we used antibodies against bone morphogenetic protein-2 (BMP-2; Austral Biologicals, San Ramon, CA, USA) at a dilution of 1:200, osteocalcin (a gift from Dr. S. Ishizuka, Teijin Biomedical Research Institute, Tokyo) at a dilution of 1:100, osteopontin (MAP, LSL) at a dilution of 1:100 with PBS, or rabbit anti-bovine type II collagen (LSL) at a dilution of 1:200 for 1.5 h at 37°C. After washing, the specimens were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG second antibodies (CAPPEL; Organon Teknika, Durham, NC, USA, 1:500 dilution) for 1 h at 37°C. Immunoreactivity was visualized with a diaminobenzidine (DAB) kit (Sigma) for 5–10 min, and then counterstained lightly with hematoxylin. Control cultures were incubated directly with the secondary antibodies in the absence of the primary antibodies and processed as above. No significant positive immunoreactivity was found in controls.

Transplantation
Recipient mice were anesthetized by an intraperitoneal injection of nembutal (0.6 mg/10 g body weight), as previously reported in detail (Ishizeki et al., 1987). Cell pellets were collected using a Venula-V-2 needle (Top Co. Ltd, Tokyo) and were transplanted into two sites in the spleen. Prior to extraction of the explants, the mice underwent vascular perfusion with 4% paraformaldehyde in PBS (pH 7.2).

Histological and histochemical observations
von Kossa staining
Explants transplanted for 1 and 4 weeks were stained using the von Kossa stain for the histochemical analysis of mineralization. Briefly, the specimens surrounded by splenic tissue were fixed with 4% paraformaldehyde, washed thoroughly in PBS, and incubated with 5% silver nitrate for 20 min for von Kossa staining (Ishizeki et al., 1996b).

Light and electron microscopy
For light microscopic observations, undecalcified semithin sections (1 μm) after 1 week of transplantation and decalcified semithin sections after 4 weeks of transplantation were prepared from samples embedded in epoxy-resin (Epon-812; Taab Laboratories Equipment Ltd, Aldermaston, UK) and stained with 0.1% toluidine blue (pH 3.0).

For electron microscopy, samples transplanted for 2 and 4 weeks were fixed in cold buffered 2.5% glutaraldehyde (pH 7.2) for 2–4 h and post-fixed in 1% osmium tetroxide, according to conventional procedures. After dehydration through a graded ethanol series, the specimens were embedded in Epon 812. Ultrathin sections without decalcification were cut with a diamond knife using an LKB-8800 ultratome (Bromma, Sweden). These sections were then stained with uranyl acetate and lead citrate prior to examination under an H-7100 (Hitachi, Tokyo) electron microscope.

Immunohistochemical procedures
Explants for immunostaining were harvested after 1, 2, and 4 weeks of transplantation and fixed in 4% paraformaldehyde for 30 min. The specimens at 4 weeks of transplantation were further decalcified for 2-5 days in PBS (pH 7.2) containing 4% EDTA. After washing with PBS, the explants were embedded in Tissue-Tek Compound 4583 (Sakura Finetechnical Co., Tokyo) prior to cutting with a cryostat. Cryosections at a 6-μm thickness were obtained from the frozen samples at -20°C, washed thoroughly with PBS, and then processed for examination after immunofluorescence and immunoperoxidase staining.

Types I, II, and X collagens were examined by immunoperoxidase staining to identify collagenous proteins in the explants. Slides were reactivated using a STUF MARK 2 unmasking solution kit (Serotec, Inc., Raleigh, NC, USA) for 10 min, and endogenous peroxidase was blocked by treatment with 3% H2O2 in methanol for 30 min at room temperature. After washing with PBS, specimens were incubated with rabbit anti-rat type I collagen antibody (LSL Co. Ltd., Tokyo) diluted 1:200 in PBS containing 0.1% bovine serum albumin, rabbit anti-rat type X collagen antibody (MAP, LSL) diluted 1:100 with PBS, or rabbit anti-bovine type II collagen (LSL) at a dilution of 1:200 for 1.5 h at 37°C. After washing, the specimens were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG second antibodies (CAPPEL; Organon Teknika, Durham, NC, USA, 1:500 dilution) for 1 h at 37°C. Immunoreactivity was visualized with a diaminobenzidine (DAB) kit (Sigma) for 5–10 min, and then counterstained lightly with hematoxylin. Control cultures were incubated directly with the secondary antibodies in the absence of the primary antibodies and processed as above. No significant positive immunoreactivity was found in controls.

For immunofluorescence staining, we used antibodies against bone morphogenetic protein-2 (BMP-2; Austral Biologicals, San Ramon, CA, USA) at a dilution of 1:200, osteocalcin (a gift from Dr. S. Ishizuka, Teijin Biomedical Research Institute, Tokyo) at a dilution of 1:100, osteopontin (MAP, LSL) at a dilution of 1:100 with PBS, or rabbit anti-bovine type II collagen (LSL) at a dilution of 1:200 for 1.5 h at 37°C. After washing, the specimens were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG second antibodies (CAPPEL; Organon Teknika, Durham, NC, USA, 1:500 dilution) for 1 h at 37°C. Immunoreactivity was visualized with a diaminobenzidine (DAB) kit (Sigma) for 5–10 min, and then counterstained lightly with hematoxylin. Control cultures were incubated directly with the secondary antibodies in the absence of the primary antibodies and processed as above. No significant positive immunoreactivity was found in controls.

For immunofluorescence staining, we used antibodies against bone morphogenetic protein-2 (BMP-2; Austral Biologicals, San Ramon, CA, USA) at a dilution of 1:200, osteocalcin (a gift from Dr. S. Ishizuka, Teijin Biomedical Research Institute, Tokyo) at a dilution of 1:100, osteopontin (MAP, LSL) at a dilution of 1:100, and osteonectin (LSL) at a dilution of 1:50 to identify bone-type marker proteins. After a thorough washing with PBS, the cryosections were incubated with polyclonal rabbit antibodies against BMP-2, osteocalcin, osteopontin, and osteonectin for 2 h at 37°C, washed three times with PBS, and incubated with an Alexa 488-conjugated antibody (Molecular Probes, Inc., Eugene, OR, USA) for 1 h at 37°C. Control sections were treated with the Alexa-labeled secondary antibody alone. The specimens were mounted in a mixture of glycerol and PBS (9:1, v/v) and observed using a fluorescence confocal laser microscope (LSM-510; Carl Zeiss, Munich, Germany).
The von Kossa staining was performed at 1 and 4 weeks of transplantation in order to evaluate calcium deposition. In sections at 1 week after transplantation, matrix calcification was detected as small spot-like deposits around the chondrocytes (Fig. 2A). The matrix calcification appeared as irregularly expanded deposits, and some specimens contained small osteocyte-like cells in the pellets. The von Kossa staining confirmed that the dark areas seen by phase-contrast microscopy coincided with the calcification of the extracellular matrix surrounding the cell pellets (Fig. 2B).

Electron probe microanalysis
The components of the calcified matrix at 4 weeks after transplantation were analyzed using electron probe microanalysis, which revealed the presence of calcium and phosphorus (Fig. 3). These components were consistent with those found in the intact bone matrix.

Electron microscopy
After 2 weeks of transplantation, the chondrocytes in the uncalcified regions of the cell pellets contained
Expression of osteogenic proteins in chondrocytes

large, ovoid nuclei and some organelles, including mitochondria. It was notable that the pericellular matrix at this stage contained newly formed thick-banded collagen fibrils, intermingled with the initial fine, cartilage-specific collagenous fibrils (Fig. 4A).

After 4 weeks of transplantation, many small ovoid cells appeared in the calcified and uncalcified regions of the cell pellets (Fig. 4B, inset). The osteocytic cells were ultrastructurally characterized by an increase in dense bodies and small mitochondria, and by elongated cytoplasmic processes penetrating into the calcified extracellular matrix (Fig. 4B). These morphological appearances strongly resembled those of osteocytes in the intact bone matrix.

Fig. 2. von Kossa staining after 1 and 4 weeks of transplantation. A: A small amount of matrix calcification is initiated in the explant at 1 week of transplantation. B: Specimen transplanted for 4 weeks reveals intense matrix calcification. Scale bars = 10 μm (A) and 100 μm (B)

Fig. 3. Electron probe microanalysis reveals the presence of calcium and phosphorus peaks in the extracellular matrix at 4 weeks of transplantation.
**Immunoperoxidase staining**

Type II collagen was distributed throughout the extracellular matrix after 1 week of transplantation (Fig. 5A). This immunoreactivity was in agreement with the synthesis period of matrix formation by chondrocytes and confirmed that the transplanted chondrocytes synthesized type II collagen during the early periods of transplantation. However, the immunoreactivity gradually decreased with the duration of transplantation, probably accompanied by the synthesis of type I collagen (Fig. 5B).

Type X collagen is the major marker collagen characterizing hypertrophic chondrocytes. The expression of type X collagen was localized around hypertrophic cells after 1 week of transplantation (Fig. 5C), but its immunoreactivity was absent in the peripheral regions of the osteocytic masses (Fig. 5D). This finding indicates that some cells converted directly into osteocyte-like cells, while some of the transplanted chondrocytes become type X collagen-positive hypertrophic cells.

Immunoreactivity for type I collagen was undetectable in the cell pellets at 1 week of transplantation (Fig. 5E), but specimens harvested after 4 weeks showed type I collagen synthesis.
Fig. 5. Legend on the opposite page.
the calcified matrix masses contained osteocytic cells immunopositive for osteocalcin (Fig. 6B). The bone marker protein, osteocalcin, was therefore shown to be synthesized by osteocytic cells prior to the initiation of calcification. Osteopontin and osteonectin were immunolocalized in the peripheral zone of the lacunae surrounding small round cells, presumably transformed from chondrocytes (Fig. 6C, D). Osteonectin expression was observed throughout the expansion period of collagen distributed in association with small round cells and along the inner wall of the calcified matrix encircling those cells (Fig. 5F).

**Immunofluorescence staining**

Immunofluorescence staining revealed BMP-2 within the calcified matrix (Fig. 6A). Immunofluorescence staining also indicated that, after 4 weeks of transplantation, the calcified matrix masses contained osteocytic cells immunopositive for osteocalcin (Fig. 6B). The bone marker protein, osteocalcin, was therefore shown to be synthesized by osteocytic cells prior to the initiation of calcification. Osteopontin and osteonectin were immunolocalized in the peripheral zone of the lacunae surrounding small round cells, presumably transformed from chondrocytes (Fig. 6C, D). Osteonectin expression was observed throughout the expansion period of

Fig. 6. Immunofluorescence staining for bone-marker proteins at 4 weeks of transplantation. A: Bone morphogenetic protein-2 (BMP-2) is intensely immunolocalized in the basket-like calcified extracellular matrix. B: Osteocalcin (OC) is detected in the calcified matrix, similar to the distribution for BMP-2. C: Immunoreactivity for osteopontin (OP) occurs in ovoid osteocytic cells. D: Osteonectin (ON) is immunostained at the periphery of small round osteocytic cells embedded in the calcified matrix. Scale bars = 100 μm
transplantation.

Figure 7 shows the expression of cartilage- and bone-type proteins during the transplantation of chondrocyte pellets, as evaluated by histological and immunohistochemical analyses. The cells switched from expressing a cartilaginous phenotype during the early period of transplantation to an osteogenic phenotype during the third to fourth weeks of transplantation.

Discussion

We have demonstrated that cell pellets consisting of Meckel's chondrocytes demonstrated osteogenic potential at the cellular level when they were transplanted into the isogenic mouse spleen. Transplanted cell pellets aggregated with each other and produced metachromatic cartilage matrices. These matrices were not resorbed by osteoclasts or macrophages, and subsequently formed calcified matrices. Since the appearance of host-originated bone forming cells such as osteoblasts was not observed around the calcified matrices, the newly-formed bone-like matrices seemed to arise from the cells transformed into osteogenic cells from chondrocytes. This evidence was further supported by immunohistochemical analyses, showing that the expression of the cartilage-specific type II collagen decreased while the expression of bone-type proteins increased. This confirmed that the cellular transformation of Meckel's chondrocytes could occur under in vivo conditions, in agreement with previous reports describing the transformation of intact Meckel's cartilage into osteogenic cell types synthesizing bone-type collagen (Ishizeki et al., 1992a).

The cells located in the central zone of 4-week transplanted specimens were surrounded by thick-banded collagen fibrils formed at the inner wall of the lacunae. Thick-banded collagen fibrils were easily distinguishable from the fine, type II collagen fibrils existing in the intact cartilage matrix. These fibrils ultrastructurally resembled those reported during the transformation of Meckel's chondrocytes in a cell culture (Ishizeki et al., 1996b, 1998a) and in the femur growth plate of chick embryos (Roach et al., 1995; Roach 1997). They were identified as type I collagen in a bone-composing collagen matrix. Furthermore, immunohistochemical analysis indicated that type I collagen was not present in the cell pellets but was widely distributed in the calcified matrix during the late period of transplantation. This suggests that a switch in fibrillogenesis to type I collagen is involved in the osteogenic transformation of chondrocytes and is therefore indicative of osteogenic transformation. Biochemical studies of cellular transformation also provide evidence for the transformation of chondrocytes by demonstrating the expression of marker genes for bone (Celeste et al., 1986; Weiss et al., 1986; Lian et al., 1989; Strauss et al., 1990; Descalzi-Cancedda et al., 1992). Inoue et al. (1995) observed the biphasic expression of type I collagen mRNA that peaked at the start of culture and the beginning of mineralization during the cell culture of rat condylar cartilage. They suggested that the second peak of type I collagen may have been related to the osteogenic differentiation of mandibular condyle cells. In the present study, we thoroughly removed the perichondrium of Meckel's cartilage prior to transplantation, and thick banded- and immuno-positive type I collagen, which was identified ultrastructurally and immunocytochemically, must therefore have been newly produced by osteogenic cells transdifferentiated directly from chondrocytes. Type X collagen is a collagen specific to hypertrophic chondrocytes. We have confirmed that Meckel's chondrocytes in vitro express type X collagen and that type X collagen-positive hypertrophic chondrocytes switch to an osteogenic phenotype (Ishizeki et al., 1997). However, after transplantation in the present study, not only type X collagen-positive chondrocytes but also non-hypertrophic typical chondrocytes, transformed into bone protein-expressing cells. Environmental stimuli in the spleen may have induced the transformation from type X collagen-negative chondrocytes to osteogenic cells.
Further evidence for cellular transformation was provided by immunohistochemical analysis for bone-type marker proteins. Although osteocalcin, which was observed coincident with the appearance of type X collagen expression, shows a high affinity for hydroxyapatite crystals and has been used as a principal bone matrix marker protein, it has been also detected in calcified cartilage, dentin, and cementum (Hauschka, 1977; Hauschka and Reid, 1978; Glimcher et al., 1979; Linde et al., 1982; Ishizeki et al., 1996c). Thus, the transformation of chondrocytes in the present transplant experiments cannot be proven by the expression of osteocalcin alone, but the sequential expression of other bone-associated proteins—BMP-2, osteopontin, and osteonectin—strongly suggest the osteocytic transformation of chondrocytes. BMP-2 is secreted by osteoblasts and osteocytes and is a bone-specific protein. BMP-2 expression was widely distributed throughout the calcified extracellular matrix after 4 weeks of transplantation. Osteonectin was expressed in the peripheral zone of cells for the longest period, differing from the expression patterns of osteocalcin and BMP-2 in the calcified matrix. The source of these proteins appeared to be through their synthesis and secretion by cells that had acquired an osteogenic phenotype. Osteopontin is a major non-collagenous protein in the bone matrix and is expressed by bone-related cells including osteoclasts, osteoblasts, and osteocytes (Nomura et al., 1993; Nanci et al., 1996). In the present study, osteopontin expression was recognized in the calcified matrix and in transforming cells after 4 weeks of transplantation. This immunoreactivity for osteopontin was consistent with the appearance of osteocytic cells in cultures of Meckel's cartilage (Ishizeki et al., 1998b), and provides further evidence of transformation of transplanted chondrocytes with the acquisition of osteopontin-positive features by phenotypic switching.

Although there are some reports on the transformation of chondrocytes in growth plate cartilage of long bones in vivo (Holtrop, 1972; Yoshioka and Yagi, 1988; Galotto et al., 1994; Roach et al., 1995), it is of interest that ectomesenchymal cells originating in the neural crest, such as the chicken chondroid bone (Hall, 1972; Beresford, 1981; Lengelé et al., 1996) and Meckel's cartilage (Ishizeki et al., 1996b, 1997) as well as mandibular condylar chondrocytes, demonstrate high osteogenic potential in comparison with those of mesoderm-derived cartilage. Chondroid bones appear in some developing membranous bones, such as cranial, lower facial, and mandibular bones (Goret-Nicaise and Dhem, 1982). This tissue develops as an intermediate tissue of cartilage and bone: the cells are larger than osteogenic cells and express some chondrogenic markers such as type II collagen, but its extracellular matrix appears bone-like by staining for proteoglycans (Lengelé et al., 1996). Some investigators including Enlow (1962), Hall (1972), and Luder and Schroeder (1992) have suggested the possibility that the cells in the chondroid bone transdifferentiate into osteogenic cells. Mizoguchi et al. (1997) detected the expression of types I, II, and X collagen as well as osteocalcin in the chondroid bone matrix of rat glenoid fossa using immunocytochemistry, and reported that, since neither the active deposition of a bone matrix nor intracellular labeling for osteocalcin were observed, the cells within the chondroid bone apparently displayed osteocytic, rather than osteoblastic, characteristics. These reports further support our hypothesis that neural crest-derived ectomesenchymal cells have a higher potential than mesoderm-derived cells for cellular transformation into bone-forming cells in response to altered environmental stimuli.

In conclusion, the results of the present study suggest that the phenotypic transformation of Meckel's chondrocytes can occur under in vivo culture conditions at a cellular morphological level. Further studies are required to ascertain if such features are an intrinsic capacity of ectomesenchyme-derived cartilage.

References

Ben-Ami YK, von der Mark K, Franzen A, Bernard BD, Lunazz GC, Silbermann M: Transformation of fetal secondary cartilage into embryonic bone in organ cultures of human mandibular condyle. Cell Tissue Res 271: 317-3322 (1993).

Beresford WA: Chondroid bone, secondary cartilage and metaplasia. 1st ed., Urban & Schwarzenberg, Baltimore, 1981.

Celeste AJ, Rosen V, Bueck JL, Kriz R, Wang EA, Wozeny JM: Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J 5: 1885-1890 (1986).

Descalzi-Cancedda F, Gentili C, Manduca P, Cancedda R: Hypertrophic chondrocytes undergo further differentiation in culture. J Cell Biol 117: 427-435 (1992).

Enlow DH: A study of the post-natal growth and remodeling of bone. Am J Anat 110: 79-101 (1962).

Fang J, Hall BK: Chondrogenic cell differentiation from membrane bone periostea. Anat Embryol 196: 349-362 (1997).
Galotto M, Campanile G, Robino G, Descalzi-Cancedda F, Bianco P, Cancedda R: Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo. J Bone Miner Res 9: 1239-124 (1994).

Glimcher MJ, Lefteriou B, Kossida D: Identification of O-phosphoserine, and γ-carboxyglutamatic acid in the non-collagenous proteins of bovine cementum: comparison with dentin enamel and bone. Calcif Tissue Int 28: 83-86 (1979).

Goret-Nicaise M, Dhem A: Presence of chondroid tissue in the symphyseal region of the growth human mandible. Acta Anat 113: 189-195 (1982).

Hall BK: Immobilization and cartilage transformation into bone in the embryonic chick. Anat Rec 173: 391-404 (1972).

Hall BK: Bones and cartilage: developmental and evolutionary skeletal biology. 1st ed, Elsevier Academic Press, London, 2005.

Harada Y, Ishizeki K: Evidence for transformation of chondrocytes and site-specific resorption during the degradation of Meckel's cartilage. Anat Embryol 197: 439-450 (1998).

Hauschka PV: Quantitative determination of γ-carboxyglutamatic acid in proteins. Analit Biochem 80: 212-223 (1977).

Hauschka PV, Reid ML: Timed appearance of a calcium-binding protein containing γ-carboxyglutamatic acid in developing chick bone. Devel Biol 65: 426-434 (1978).

Holtrop ME: The ultrastructure of the epiphyseal plate. II. The hypertrophic chondrocytes. Calcif Tissue Res 9: 140-151 (1972).

Inoue H, Nebgen D, Veis A: Changes in phenotypic gene expression in rat mandibular condylar cartilage cells during long-term culture. J Bone Miner Res 10: 1691-1697 (1995).

Ishizeki K, Fujiwara N, Skakura Y, Nawa T: Evidence for several features of chondrocytes in vitro. Calcif Tissue Int 38: 155-162 (1986).

Ishizeki K, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G: Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc Natl Acad Sci USA 86: 1143-1147 (1989).

Jee WSS: The skeletal tissues. In: Cell and tissue biology: a textbook of histology. 6th ed (Weiss L ed), Urban & Schwarzenberg, Baltimore, 1988 (p. 213-217).

Lengelé B, Schowing J, Dhem A: Chondroid tissue in the early facial morphogenesis of the chick embryo. Anat Embryol 193: 505-513 (1996).

Lewinson D, Silbermann M: Parathyroid hormone stimulates proliferation of chondroprogenitor cells in vitro. Calcif Tissue Int 38: 28: 83-86 (1979).

Linde A, Bhown M, Cothran WC, Hoglund A, Butler WT: Evidence for several γ-carboxyglutamatic acid-containing proteins in dentin. Biochem Biophys Acta 704: 235-239 (1982).

Luder HU, Schroeder HE: Light and electron microscopic morphology of the temporomandibular joint in growing and mature crab-eating monkys (Macaca fascicularis): the condylar calcified cartilage. Anat Embryol 185: 189-199 (1992).
Mizoguchi I, Takahashi I, Sasano Y, Kagayama M, Kuboki Y, Mitani H: Localization of types I, II and X collagen and osteocalcin in intramembranous, endochondral and chondroid bone of rats. Anat Embryol 196: 291-297 (1997).

Roach HI, Erenpreisa J, Aigner T: Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. J Cell Biol 131: 483-494 (1995).

Roach HI: New aspect of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J Bone Miner Res 12: 795-805 (1997).

Schmidt J, Livne E, Erfle V, Gossner W, Silbermann M: Morphology and in vivo growth characteristics of an atypical murine proliferative osseous lesion induced in vitro. Cancer Res 46: 3090-3098 (1986).

Silbermann M, Frommer J: The nature of endochondral ossification in the mandibular condyle of the mouse. Anat Rec 172: 659-668 (1972).

Silbermann M, Frommer J: Ultrastructure of developing cartilage in the mandibular condyle of the mouse. Acta Anat 90: 330-34 (1974).

Strauss PG, Closs EI, Schmidt J, Erfle V: Gene expression during osteogenic differentiation in mandibular condyles in vitro. J Cell Biol 110: 1369-1378 (1990).

Weiss A, von der Mark K, Silbermann M: A tissue culture system supporting cartilage cell differentiation, extracellular mineralization, and subsequent bone formation, using mouse condylar progenitor cells. Cell Differ 19: 103-113 (1986).

Yoon K, Rutledge SJC, Buenega RF, Rodan G: Characterization of the rat osteocalcin gene: stimulation of promoter activity by 1, 25-dihydroxyvitamin D3. Biochemistry 27: 8521-8526 (1988).

Yoshioka C, Yagai T: The fate of hypertrophic chondrocytes. (in Japanese). Bone Metab 13: 257-258 (1980).

Yoshioka C, Yagai T: Electron microscopic observations on the fate of hypertrophic chondrocytes in condylar cartilage of rat mandible. J Craniof Genet Dev Biol 8: 253-264 (1988).

Nanci A, Zalzal S, Gotoh Y, McKee MD: Ultrastructural characterization and immunolocalization of osteopontin in rat calvarial osteoblast primary cultures. Microsc Res Tech 33: 214-231 (1996).

Nomura S, Hirakawa K, Nagoshi J, Hirota S, Kim H-M, Takemura T, Nakase N, Takaoka K, Mastumoto S, Nakajima Y, Takebayashi K, Takano-Yamamoto T, Ikeda T, Kitamura Y: Method for detecting the expression of bone matrix protein by in situ hybridization using decalcified mineralized tissue. Acat Histochem Cytochem 26: 303-309 (1993).