Xiphoid Process-Derived Chondrocytes: A Novel Cell Source for Elastic Cartilage Regeneration

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ABSTRACT

Reconstruction of elastic cartilage requires a source of chondrocytes that display a reliable differentiation tendency. Predetermined tissue progenitor cells are ideal candidates for meeting this need; however, it is difficult to obtain donor elastic cartilage tissue because most elastic cartilage serves important functions or forms external structures, making these tissues dispensable. We found vestigial cartilage tissue in xiphoid processes and characterized it as hyaline cartilage in the proximal region and elastic cartilage in the distal region. Xiphoid process-derived chondrocytes (XCs) showed superb in vitro expansion ability based on colony-forming unit fibroblast assays, cell yield, and cumulative cell growth. On induction of differentiation into mesenchymal lineages, XCs showed a strong tendency toward chondrogenic differentiation. An examination of the tissue-specific regeneration capacity of XCs in a subcutaneous-transplantation model and autologous chondrocyte implantation model confirmed reliable regeneration of elastic cartilage regardless of the implantation environment. On the basis of these observations, we conclude that xiphoid process cartilage, the only elastic cartilage tissue source that can be obtained without destroying external shape or function, is a source of elastic chondrocytes that show superb in vitro expansion and reliable differentiation capacity. These findings indicate that XCs could be a valuable cell source for reconstruction of elastic cartilage. STEM CELLS TRANSLATIONAL MEDICINE 2014;3:1381–1391

INTRODUCTION

Cartilage is located throughout the body and plays multiple important roles. Cartilage can be classified as “hyaline cartilage,” “elastic cartilage,” and “fibrocartilage” based on extracellular matrix (ECM) composition and histological morphology. The characteristics of these different types of cartilage vary in response to physiological environments, site-specific mechanical conditions, and/or developmental events, which collectively define the unique tissue property of a given anatomical site. The fact that a single type of cell—the chondrocyte—composes each type of cartilage tissue highlights the importance of cell source for the reconstruction of specific types of cartilage.

Hyaline and elastic chondrocytes cultured in vitro exhibit different characteristics, especially in terms of ECM production [1, 2]. The traits of each type of chondrocyte, such as the synthesis of function-specific ECM and proliferation tendency, affect the specific characteristics of each type of cartilage [3–5]. Accordingly, the characteristics of cells used as a source for cartilage regeneration can profoundly affect the properties of the resultant regenerated tissue. Consequently, the proper reconstruction of elastic cartilage requires chondrocytes from elastic cartilage.

Elastic cartilage can be found in auricular cartilage and consists of large lacunae of chondrocytes surrounded by elastic fibers in the pericellular area. Elastic cartilage shows expression of elastin in addition to type II collagen, which provides greater flexibility and thus the ability to withstand repeated bending. Reconstruction of human elastic cartilage usually involves large area defects, as exemplified in cases such as microtia and anotia, Treacher Collins syndrome, and acrofacial dysostosis (Nager syndrome) [6]. The recommended approach for reconstructing elastic cartilage is autologous chondrocyte-based tissue engineering, which is considered the safest and most efficient approach [7–9]. However, sources of autologous elastic cartilage are severely limited. Almost all known elastic cartilage plays an essential role in the body because of its function or by virtue of its structural importance, as in the case of auricular cartilage and epiglottal cartilage [10].

The possibility of using stem cells such as bone marrow stromal cells (BMSCs) for elastic cartilage reconstruction has been considered and explored. However, several critical problems are associated with this approach, including low chondrogenic potential owing to poor matrix production and a tendency toward vascularization and hypertrophic transition [10–16]. Using hyaline cartilage, such as costal cartilage, for elastic...
cartilage reconstruction (the most common approach) is also problematic because it forms tissue with less elastic fiber, different stiffness, and low resistance to deformation [10].

In this study, we examined a small region of cartilaginous tissue at the xiphoid process for cartilage type. The xiphoid process, also known as the xiphosternum, is vestigial cartilage attached to the lowest end of the sternum body located in the inner side of the peritoneum. Accordingly, xiphoid process cartilage can be removed without impairing any biological function, and it is the only elastic cartilage tissue source that can be obtained without destroying external shape or function. By comparing xiphoid process cartilage with other cartilages of known type, we determined that the proximal part of the xiphoid process is composed of hyaline cartilage and the distal part is composed of elastic cartilage. On the basis of these observations, we isolated chondrocytes from only the distal part of the xiphoid process, yielding elastic cartilage chondrocytes, and examined their characteristics. We found that xiphoid process-derived chondrocytes (XCs) exhibited superb in vitro expansion capacity. Moreover, when subcutaneously transplanted, XCs showed obvious characteristics of elastic cartilage, based on lacunae morphology and elastin expression at the pericellular area. Although this is basic research, studied only with animal, the results suggest that the xiphoid process is a promising source of chondrocytes for the reconstruction of elastic cartilage using a tissue-engineering approach.

**Materials and Methods**

**Chemicals**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com) unless otherwise indicated.

**Animals**

Male Sprague-Dawley rats (Central Laboratory Animal Inc., Seoul, Korea, http://www.labanimal.co.kr), maintained under standard conditions and fed standard chow and water ad libitum, were used for primary culture of chondrocytes and BMSCs. Rats weighing <250 g (<7–8 weeks old) were considered young, and rats with an average weight of 400 g (>3 months old) were considered adult. Immuno-compromised, 6- to 8-week-old mice (BALB/cSLC-Foxn1-/-; Central Laboratory Animal Inc.) were used as recipients of subcutaneous transplants. Female New Zealand white rabbits (DooYeo Biotech, Seoul, Korea, http://dybiotech.co.kr) were used at a density of 5 × 10^5 cells per 100-mm dish and cultured for 3 days. After 3 days of culture, cells were detached using 0.25% trypsin/EDTA, and viable cells were counted on the basis of trypan blue staining. After counting, each mouse was anesthetized with pentobarbital sodium (passage 0 to passage 8) at a density of 5 × 10^6 cells per 100-mm dish and cultured for 3 days. Each mouse was anesthetized with pentobarbital sodium (passage 0 to passage 8) at a density of 5 × 10^6 cells per 100-mm dish and cultured for 3 days. On reaching confluence, cells were detached using 0.25% trypsin/EDTA and subcultured at a density of 5 × 10^5 cells per 100-mm dish. For calculation of expansion rate, articular cartilage-derived chondrocytes (ACs), XCs, and costal cartilage-derived chondrocytes (CCs) from Sprague-Dawley rats were plated at each passage (passage 0 to passage 8) at a density of 5 × 10^5 cells per 100-mm dish and cultured for 3 days. After 3 days of culture, cells were detached using 0.25% trypsin/EDTA, and viable cells were counted on the basis of trypan blue staining. After counting, each type of chondrocyte was seeded on 100-mm cell strainer (BD Falcon, Franklin Lakes, NJ, http://www.corning.com) to remove undigested tissues. The number of viable cells was counted at this point by trypan blue staining to compare initial cell yield. Chondrocytes were then plated at a density of 5 × 10^3 cells per 100-mm culture dish with 2 mML-glutamine (Welgene), 1% penicillin/streptomycin (Welgene Inc., Daegu, Korea, http://www.welgene.com/mall/index.php), Masson Trichrome, Safranin O, Toluidine blue, and Alcian blue for identification of histological features. For immunohistochemical staining, deparaffinized sections were rehydrated with PBS before quenching endogenous peroxidase activity with hydrogen peroxide. Sections were blocked in PBS containing 20% normal goat serum (Dako, Glostrup, Denmark, http://www.dako.com) and then incubated with primary antibodies against type II collagen (Abcam, Cambridge, U.K., http://www.abcam.com), type I collagen (Abcam), or elastin (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com) overnight at 4°C. After incubation with primary antibody, sections were washed three times with PBS and incubated with diluted horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com). Immunoreactive proteins were detected by incubating sections with 3,3'-diaminobenzidine tetrahydrochloride (DAB) using a DAB substrate kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Sections were counterstained with fast red and observed under a light microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com).

**Cell Isolation and Culture**

Primary Culture of Chondrocytes

Xiphoid process cartilage was obtained from female New Zealand white rabbits (average weight: 2 kg). For a comparative study, articular cartilage, costal cartilage, and xiphoid process cartilage were obtained from the femoral heads, lower false rib, and distal half of the xiphoid process, respectively, of Sprague-Dawley rats (average weight: 250 g). The perichondrium was removed from each cartilage tissue, weighed, and minced into pieces 1–2 mm^3 in size. The minced cartilage particles were digested overnight in 0.5% collagenase type I (Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) at 37°C in a 5% CO2 atmosphere. Digested cartilage was then filtered through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ, http://www.corning.com) to remove undigested tissues. The number of viable cells was counted at this point by trypan blue staining to compare initial cell yield. Chondrocytes were then plated at a density of 5 × 10^3 cells per 100-mm culture dish with 2 mML-glutamine (Welgene), 1% penicillin/streptomycin (Welgene Inc., Daegu, Korea, http://www.welgene.com/mall/index.php), 2 mM-L-glutamine (Welgene), 10^-8 M dexamethasone, and 10^-4 M ascorbic acid at 37°C in a humidified atmosphere of 5% CO2. The culture medium was changed every other day. On reaching confluence, cells were detached using 0.25% trypsin/EDTA (Welgene) and then subcultured at a density of 5 × 10^5 cells per 100-mm culture dish. For calculation of expansion rate, articular cartilage-derived chondrocytes (ACs), XCs, and costal cartilage-derived chondrocytes (CCs) from Sprague-Dawley rats were plated at each passage (passage 0 to passage 8) at a density of 5 × 10^5 cells per 100-mm dish and cultured for 3 days. After 3 days of culture, cells were detached using 0.25% trypsin/EDTA, and viable cells were counted on the basis of trypan blue staining. After counting, each type of chondrocyte was seeded on 100-mm culture dishes at a density of 5 × 10^5 cells per 100-mm dish. The accumulated
expansion at each passage was calculated and presented graphically.

Primary Culture of BMSCs

Rat BMSCs were isolated and cultured, as described previously [16]. Briefly, femurs were obtained from a 250–350-g Sprague-Dawley rat, and the bone marrow was flushed with α-MEM using a syringe and 22-gauge needle. Flushed bone marrow was vigorously pipetted several times to obtain a single-cell suspension and passed through a 70-μm cell strainer before seeding in standard media at a density of 1 × 10^5 cells per 75-cm² flask (BD Falcon) for expansion. On reaching confluence, cells were detached using 0.25% trypsin/EDTA and then subcultured at a density of 5 × 10^5 cells per 100-mm tissue culture dish.

In Vitro Adipogenic Differentiation

Adipogenic differentiation potential of BMSCs and XCs was confirmed using a previously described induction procedure [17, 18]. Briefly, BMSCs and XCs at passage 5 were seeded onto a 6-well culture plate (Corning Inc., Corning, NY, http://www.corning.com) at a density of 5 × 10^5 cells per well and cultured in standard media for 2 days. The cells were then incubated in adipogenic induction media composed of high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 1% penicillin/streptomycin, 10% FBS, 10 μg/ml insulin, 10^{-7} M dexamethasone, 0.2 mM indomethacin, and 0.5 mM 3-isobutyl-1-methylxanthine for 3 days. This was immediately followed by a second cycle of adipogenic induction in which cells were cultured for an additional 2 days in adipogenic maintenance media composed of high-glucose DMEM supplemented with 1% penicillin/streptomycin, 10% FBS, and 10 μg/ml insulin. After two cycles of adipogenic induction culture, cells were fixed with 4% PFA in PBS, and lipid droplets were visualized by Oil Red O staining.

In Vitro Chondrogenic Differentiation

Chondrogenic differentiation potentials of BMSCs and XCs were confirmed using a previously described procedure [2, 17, 18]. Briefly, BMSCs and XCs were trypsinized, and 5 × 10^5 cells were centrifuged at 600 g for 20 minutes in a 15-ml conical tube (BD Falcon) in standard media. After overnight incubation, the medium was changed to chondrogenic medium consisting of high-glucose DMEM supplemented with 1% penicillin/streptomycin, 0.3 mM ascorbic acid, 0.35 mM proline, 1× ITS+3, 10^{-7} M dexamethasone, and 10 ng/ml transforming growth factor-β3 (PanGen Biotech, Suwon, Korea, http://www.pangen.com). Chondrogenic media were changed every other day. Pellets were fixed with 4% PFA in PBS after 3 weeks of in vitro chondrogenesis. Glycosaminoglycan (GAG) production was confirmed by Safranin O staining.

Subcutaneous Transplantation for In Vivo Osteogenesis

Rat BMSCs or XCs (2 × 10^6 cells per transplant) were mixed with 40 mg of macroporous biphasic calcium phosphate consisting of a 60:40 ratio of hydroxyapatite/β-tricalcium phosphate (HA/TCP) (size range: 0.5–1 mm; Biomatlante, Bretagne, France). After 2 hours of incubation, cells and HA/TCP mixtures were subcutaneously transplanted into 6- to 8-week-old immunocompromised mice (BALB/cSLC-Foxn1^-/-^). The transplants were harvested 6 weeks or 10 weeks after transplantation and fixed with 4% PFA. Transplants were decalcified in 0.25 M EDTA and embedded in paraffin blocks. The blocks were cut at a thickness of 5–8 μm and subjected to histological analysis.

Colonizing Unit Fibroblast Assay

Single-cell suspensions of chondrocytes obtained by collagenase digestion (passage 0) were counted and plated at two different densities: 1 × 10^3 and 3 × 10^3 cells per 25-cm² flask (BD Falcon). Seeded cells were incubated at 37°C in a 5% CO₂ environment for 5 days without a media change, after which colonies were fixed with 4% PFA in PBS for 30 minutes. The fixed colonies were stained with saturated methyl violet, and colonies consisting of >50 cells were counted. Mean values and standard deviations were obtained from triplicate determinations.

Western Blot Analysis

ACs, CCs, and XCs were lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com) at passage 0. Protein concentration was determined using the bicinchoninic acid assay method (Thermo Scientific, Rockford, IL, http://www.thermoscientific.com). Proteins in lysates (10 μg per sample) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk (BD Difco, Sparks, MD, http://www.bd.com) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour and incubated overnight with antibodies specific for type II collagen, elastin (Abcam), and α-tubulin (Sigma-Aldrich) at 4°C. After washing three times with TBST, the blots were incubated for 1 hour with appropriate horseradish peroxidase-conjugated secondary antibodies. Membranes were developed using an enhanced chemiluminescence detection kit (Chromogen, Seoul, Korea) and visualized by exposure to x-ray film (AGFA, Mortsel, Belgium, http://www.agfa.com).

Determination of Default Differentiation Potential

The default differentiation tendencies of chondrocytes from different tissue sources were compared by subjecting ACs, XCs, and CCs to a previously described high-density culture method [2], with modifications. Briefly, ACs, XCs, and CCs at passage 2 were seeded at a density of 1 × 10^7 cells per well in 24-well culture plates (Corning Inc.) and cultured in standard media at 37°C in a humidified atmosphere of 5% CO₂. Media were changed every other day for 2–3 weeks without subculture. Cultured cells were washed three times with PBS and fixed with 4% PFA for 30 minutes. Cells were again washed with PBS, and their chondrogenic, osteogenic, and adipogenic potentials were evaluated by Alcian blue, alizarin red S, and Oil Red O staining, respectively.

In Vivo Chondrogenesis by Subcutaneous Transplantation

Chondrocytes from articular cartilage and xiphoid process cartilage at passage 2 were plated at a density of 1 × 10^6 cells per 100-mm tissue culture dish and cultured until reaching confluence. Thereafter, cells were maintained for 2 weeks without subculturing, and the media was changed every day; this procedure resulted in a gel-like state. The resultant gel-like cell-ECM mixtures were collected with a cell scraper (TPP, Trasadingen, Switzerland, http://www.tpp.ch) and subcutaneously transplanted into 6- to 8-week-old immunocompromised mice (BALB/cSLC-Foxn1^-/-^).
After 6 or 12 weeks in vivo, transplants were fixed in a 4% PFA solution and paraffin embedded for histologic analysis. Tissue sections, cut to a thickness of 5 μm, were subjected to H&E, Safranin O, Elastica van Gieson and immunohistochemical staining and were observed by light microscopy.

In Vivo Chondrogenesis Using an ACI Model

For the ACI model, XCs and CCs at passage 2 were individually cultured without subculture to achieve a gel-like state. Gel-like cell-ECM mixtures were collected with a cell scraper and applied autologously to each rabbit using a previously described articular cartilage defect model [8, 9]. Briefly, a cylindrical wound site in articular cartilage of the patellar groove of a New Zealand white rabbit was marked using a 5-mm-diameter biopsy punch (Kai Industries, Tokyo, Japan, http://www.kai-group.com), and a full-thickness defect was created using a microdrill (Jeung Do B&P, Seoul, Korea, http://www.jeungdo.co.kr). Gel-like cell-ECM mixtures of each autologous cell type were transplanted into the experimentally created articular defect. The rabbits were sacrificed 3 and 6 weeks after transplantation. For histological analyses, medial femoral condyle tissues were fixed in 4% PFA for 10 days, followed by decalcification in 0.25 M EDTA. After decalcification, the samples were paraffin embedded, sectioned (5-μm thickness), and subjected to H&E and Elastica van Gieson staining.

Statistical Analysis

Differences among multiple groups were analyzed using one-way analysis of variance in conjunction with a Tukey-Kramer multiple comparisons test (Instat; GraphPad Software Inc., San Diego, CA, http://www.graphpad.com). Statistical significance was accepted at $p < .05$. All experiments were repeated at least three times.

RESULTS

Xiphoid Process Cartilage Consists of Two Different Types of Cartilage

To identify the types of cartilage in xiphoid processes, we obtained transverse sections of the whole xiphoid process from rats and examined them by immunohistochemical staining for type II collagen and elastin and by Elastica van Gieson and Masson Trichrome staining (Fig. 1). Staining for type II collagen was observed at different intensities throughout the xiphoid process along the proximodistal axis, whereas elastin expression was observed only in the distal part of the tissue. Elastic fibers, visible as dark purple structures in sections stained with Elastica van Gieson, were clearly present in the middle to distal part of the xiphoid process but were absent in the proximal part. Masson Trichrome staining, which stains collagen components blue, was most intense in proximal xiphoid process cartilage and was pale in the distal part of the tissue.

A comparison of costal cartilage, xiphoid process cartilage, and auricular cartilage histology showed that the proximal part of xiphoid process cartilage was similar to hyaline cartilage with respect to lacunae morphology and the high amount of ECM in the interterritorial area. In contrast, distal xiphoid process cartilage was similar to elastic cartilage, based on the expression of elastin in the pericellular area and the scarce formation of interterritorial matrix. These results indicate that the distal part of xiphoid process cartilage possesses properties of elastic cartilage, and the proximal part possesses properties of hyaline cartilage.

Chondrocytes From Xiphoid Process Cartilage Show Traits of Tissue Progenitor Cells

To examine the differentiation characteristics of XCs, we evaluated their potential to undergo adipogenesis, chondrogenesis, and osteogenesis compared with BMSCs. XCs produced adipocytes under adipogenic conditions to a lesser extent than BMSCs (Fig. 2A), the gold standard of multipotent stem cells of the mesenchymal lineage. After 3 weeks of chondrogenic differentiation, the production of GAG-containing chondrogenic ECM by XCs was confirmed by Safranin O staining. Cartilaginous matrix formation by XCs was clearly superior to that of BMSCs, which showed barely detectable GAG-containing ECM (Fig. 2B).

Superior in vitro chondrogenic potential was also observed in tests of default differentiation tendencies. In these experiments, ACs, XCs, and CCs were seeded on 24-well plates and maintained in standard media without passage. After 2–3 weeks of culture in standard media, differentiation tendencies were confirmed by Alcian blue, alizarin red S, and Oil Red O staining. Interestingly, these chondrocytes all exhibited chondrogenic ECM production, even without chondrogenic induction. Among them, XCs showed the most intense Alcian blue staining, indicating the greatest synthesis and deposition of GAGs. Although ACs, XCs, and CCs showed chondrogenic differentiation, none of the cells differentiated along osteogenic or adipogenic lineages in the absence of induction, based on alizarin red S and Oil Red O staining (supplemental online Fig. 1).

To exclude potential ambiguities associated with in vitro osteogenesis, we tested XCs and BMSCs in an in vivo ectopic bone model. Whereas BMSCs showed bone and marrow formation, XCs showed cartilage tissue formation, even in this strongly osteogenic environment (Fig. 2C). Furthermore, the cartilage tissue formed in an in vivo ectopic bone model showed characteristics of elastic cartilage, as indicated by elastic fibers shown in Elastica van Gieson-stained section (Fig. 2Dc). Several other staining methods to visualize GAG-rich ECM did not work with elastic cartilage (Fig. 2D; supplemental online Fig. 2), as indicated by the fact that proximal and middle parts of the xiphoid process, which contains hyaline cartilaginous ECM to some degree, showed positively stained results with Safranin O, Toluidine blue, and Alcian blue staining, whereas the distal part of xiphoid process and auricular cartilage showed only a dim outline of cartilage tissue (supplemental online Fig. 2).

Good stem/progenitor cells have the capacity for density-independent growth, a property that can be tested by seeding cells at clonogenic densities and assaying for colony-forming unit fibroblast (CFU-F) formation. Cell populations that exhibit better density-independent growth tend to show better in vitro expansion. Colony-forming ability, cell yield, and cumulative cell growth of XCs were compared with those of ACs and CCs to assess the traits that influence cell expansion and growth (Fig. 3). After seeding cells at a density of $1 \times 10^5$ cells per plate, the number of colonies formed by ACs, XCs, and CCs were 15.6 ± 2.1, 57.0 ± 8.7, and 85.0 ± 10.8, respectively. When 3 × $10^5$ cells were seeded, the corresponding colony numbers were 43.3 ± 6.7, 176.6 ± 11.2, and 213.3 ± 13.1, and the proportion of CFU-Fs in ACs, XCs, and CCs was 1.44%, 5.8%, and 7.11%, respectively. XCs colony-forming efficiency and colony size were comparable to those of CCs, and superior to those of ACs (Fig. 3A, B).

The initial cell yields of ACs, XCs, and CCs were 6,818.0 ± 1,219.0, 11,790.2 ± 1,481.8, and 3,453.6 ± 800.3 cells per
milligram of tissue, respectively. XCs showed the highest initial cell yield, which was approximately 1.7- and 3.4-fold greater than those of ACs and CCs, respectively (Fig. 3C).

A comparison of cumulative cell growth among ACs, XCs, and CCs indicated that XCs showed the highest in vitro expansion ability (Fig. 3D). ACs, which showed much higher initial cell yield than CCs, showed the lowest in vitro expansion among the three chondrocyte types.

An examination of the morphology of each type of chondrocyte at each passage revealed an initial round shape that gradually converted into a fibroblastic spindle shape with successive passages. All three chondrocyte types showed a similar morphological trend from round to sharp spindle shape (supplemental online Fig. 3).

Comparison of Expressed ECM Components Between XCs and Other Hyaline Cartilage Chondrocytes

To confirm the characteristics of XCs as an elastic cartilage, we compared expression of type II collagen and elastin in XCs with that in ACs and CCs (Fig. 3E). Primary cultured chondrocytes were lysed at passage 0 and subjected to Western blot analysis. All chondrocyte types showed expression of type II collagen at passage 0, although the degree of expression differed according to the chondrocyte source. XCs showed the lowest expression of type II collagen and the highest expression of elastin compared with other chondrocytes. These data confirm that distal xiphoid process cartilage is composed of elastic cartilage.

Age-Related Characteristics of XCs

As a source of chondrocytes, it is desirable to retain progenitor cells in the tissue of adult animals. To examine age-related characteristics, we compared CFU-Fs and cell yield of XCs with those of CCs, which showed excellent colony-forming efficiency and cell expansion (Fig. 3). Whereas XCs from young rats yield a lower number of CFU-Fs than was the case for CCs, the number of CFU-Fs obtained from adult rats was higher for XCs than CCs (Fig. 4A). Cell yield decreased with age in XCs and CCs (Fig. 4B). However, the fold decrease was greater in CCs (∼3.9-fold) compared with XCs (∼2.3-fold). In young rats, the cell yield was approximately 3.4-fold higher for XCs than CCs, a difference that was even greater in adult rats (∼5.7-fold). To establish a relationship between the number of CFU-Fs and the cartilage tissue cell yield, we calculated the number of CFU-Fs per milligram of cartilage tissue (Fig. 4C). As expected, the mean CFU-F number per milligram of xiphoid process cartilage was higher than that for costal cartilage in both young and adult rats. Considering the age-related change in the cell yield and CFU-Fs, the size of the progenitor cell pool in XCs was only slightly influenced by age.

Elastic Cartilage Formation by Subcutaneously Transplanted XCs

Chondrocytes from two different type of cartilage, XCs from elastic cartilage and ACs from hyaline cartilage, were maintained in a high-density culture to form a gel-like cell-ECM mixture, as...
OR chondrogenic induction for 3 weeks transplanted into nude mice and incubated in vivo for 6 or 12 weeks. On collection of the transplanted tissues, the cartilage tissues formed by XCs were found to be larger than those formed by ACs (supplemental online Fig. 4). In vivo-incubated XC tissues showed larger lacunae and accumulated a very small amount of ECM only in pericellular areas (Fig. 5Ca–5Cd) compared with tissues formed from in vivo-incubated ACs, which showed smaller lacunae and abundant interterritorial matrix accumulation (Fig. 5Ce–5Ch). To confirm the formation of elastic cartilaginous matrix, we performed Elastica van Gieson staining and immunohistochemical staining for elastin, which revealed elastic fibers in transplants of XCs (Fig. 5Cc, 5Cd, 5D) that were absent in ACs (Fig. 5Cg, 5Ch). The presence of elastic fiber at the pericellular area and the expression of elastin revealed by histological analysis confirmed that the cartilage tissue formed by subcutaneously transplanted XCs was elastic cartilage.

**Evaluation of In Vivo Chondrogenic Differentiation in an ACI Model**

CFU-Fs were also found in rabbit xiphoid process cartilage at a frequency of 191.7 ± 11.2 per 1 × 10^6 cells (Fig. 6A). XCs and CCs were obtained from rabbits and cultured to a gel-like cell-ECM mixture for autologous application to a full-thickness rabbit ACI model, as indicated in Figure 5A. Following autologous transplantation, the newly formed cartilage tissues were compared between XC- and CC-transplanted groups. Some parts of the XC-transplanted group exhibited the lacunae morphology of elastic cartilage, whereas the CC-transplanted group showed a typical hyaline cartilage morphology. Elastica van Gieson staining demonstrated that XCs differentiated into elastic cartilage rich in elastic fibers, even in an articular cartilage regeneration environment (Fig. 6). The incidence of elastic cartilage tissue formation by XC- and CC-transplanted groups in the ACI model is summarized in Table 1. Interestingly, elastic cartilage formation was detected in more than 34% of the XC-transplant group (Table 1; supplemental online Fig. 5) but was completely absent in the CC-transplant group (Table 1; Fig. 6B). Considering that only sections in the middle of the defect were examined, it is likely that the incidence of elastic cartilage formation in the XC-transplant group is actually higher than the reported value. These results indicate that XCs have the capacity to reliably regenerate elastic cartilage, even in a hyaline cartilaginous environment.

**DISCUSSION**

This study is based only on animal models, and much work is needed to verify the possibility of translation to clinical application. Previous reports on the postnatal development of the human sternum were not thoroughly analyzed with histological methods but rather with radiographic methods. Much previous literature reports that human xiphoid process has a high degree of individual variation [19–22]. Although the tendency for calcification also shows a high degree of individual variation, O’Neal et al. reported that the site of ossification is not present in the xiphoid process until the age of 6 years and, if ever present, rarely consists of more than one ossification center [21]. The ossification center is located at the proximal end of the xiphoid process, and ossification at this site results in the fusion of the sternum and the xiphoid process. Ossification in the xiphoid process proceeds internally in the direction of the distal end. Xiphoid process from animal tissue also showed mild
calcification at the proximal portion of the xiphoid process; therefore, the tissues were decalcified with 0.25M EDTA before histological analysis. However, the primary culture of elastic chondrocytes from xiphoid process proceeded without difficulty because the distal part of the xiphoid process remains soft and flexible. Consequently, even if ossification tends to progress with age, elastic cartilage could be obtained by excising the tip of the xiphoid process through laparoscopy in young ages. In case of congenital deformity such as microtia and anotia, the deformity can be detected at birth; therefore, banking of the cells is possible even earlier, when no ossification center is present in the xiphoid process. Removal of the xiphoid process seems to leave no handicaps or other difficulties based on the fact that it is usually surgically removed in case of fracture [23].

Figure 3. Evaluation of the in vitro expansion characteristics of ACs, XCs, and CCs and extracellular matrix expression. (A, B): ACs, XCs, and CCs were plated at a density of $1 \times 10^3$ cells or $3 \times 10^3$ cells and cultured for 5 days. Colonies consisting of $>50$ cells were counted from each flask. XCs showed superior initial cell yields. Magnification $\times 40$ (C) and cumulative cell growth (D). (E): ACs, XCs, and CCs at P0 were cultured in standard media. Upon reaching confluence, cells were lysed and analyzed by Western blotting. All results are expressed as means $\pm$ SEM determined from at least three independent experiments. $***$, $p < .001$. Abbreviations: AC, articular cartilage-derived chondrocyte; CC, costal cartilage-derived chondrocyte; Col II, type II collagen; P, passage; XC, xiphoid process-derived chondrocyte.

Figure 4. Age-related characteristics of XCs. CFU-Fs (A) and cell yields (B) were compared with XCs obtained from young and adult rats. Colonies were stained with saturated methyl violet, and colonies consisting of $>50$ cells were counted. Differences between the two groups were assessed by expressing results as the number of CFU-Fs per milligram of tissue (C). Results are presented as mean $\pm$ SEM of four independent experiments. $^*$, $p < .05$; $^*$*, $p < .01$; $^{***}$, $p < .001$. Abbreviations: CC, costal cartilage-derived chondrocyte; CFU-F, colony-forming unit fibroblast; n.s., not significant; XC, xiphoid process-derived chondrocyte.
Some previous reports have suggested that the xiphoid process is composed of hyaline cartilage [13, 24]. In this study, we clearly demonstrated that the xiphoid process is composed of two different types of cartilage: hyaline cartilage in the proximal part and elastic cartilage in the distal part. The identification of cartilage types in the xiphoid process not only improves our understanding of cartilage structures in appendages but also establishes a useful tissue source for elastic cartilage regeneration.

The avascular structure and low cell density of cartilage limits tissue regeneration. Therefore, an effective tissue regeneration strategy for elastic cartilage reconstruction is needed to repair defects caused by congenital deformities or trauma. An approach...

Figure 5. Elastic cartilage regeneration in vivo. (A): Each chondrocyte type was cultured to confluence and maintained in culture for 3 weeks to form a gel-like state. (B): The characteristics of matrix formed in the gel-like state were assessed by subjecting the cells to Alcian blue, alizarin red S, and Oil Red O staining. Each chondrocyte type cultured to form a gel-like state, as shown in (B), was subcutaneously transplanted into immunocompromised mice and incubated in vivo for 12 weeks. (C): For histologic analyses, sections of transplants were stained with H&E (Ca, Ce), Safranin O (Cb, Cf), and Elastica van Gieson (Cc, Cd, Cg, Ch). (D): XC transplants were subjected to immunohistochemical staining for elastin. Scale bars = 100 μm. Abbreviations: AC, articular cartilage-derived chondrocyte; C, cartilage; CC, costal cartilage-derived chondrocyte; H&E, hematoxylin and eosin; V, vessel; XC, xiphoid process-derived chondrocyte.
cartilage-derived chondrocyte; CFU-F, colony-forming unit fibroblast; H&E, hematoxylin and eosin; XC, xiphoid process-derived chondrocyte.

transplanting each type of cell in a gel-like state into a rabbit model of full-thickness articular cartilage defect. Representative H&E and Elastica van Gieson staining of normal articular cartilage, and XC- and CC-treated groups are shown. Cartilage regenerated by XCs in the ACI model exhibited an elastic cartilage morphology, as demonstrated by the presence of large lacunae and elastic fibers. The dotted circle shows elastic cartilage formation in the XC-transplanted group. Scale bar = 100 μm. Abbreviations: ACI, autologous chondrocyte implantation; CC, costal cartilage-derived chondrocyte; CFU-F, colony-forming unit fibroblast; H&E, hematoxylin and eosin; XC, xiphoid process-derived chondrocyte.

A promising source of elastic chondrocytes has been found using cartilage tissue fragments collected from costal cartilage as a template for auricular cartilage reconstruction has been reported previously [25, 26]. However, this method has major shortcomings, such as donor site morbidity and low flexibility of the resultant tissue owing to the fact that costal cartilage is hyaline in nature. An ideal approach for elastic cartilage regeneration using a tissue-engineering approach depends on a reliable cell source and a three-dimensional construction method.

For regeneration of the outer ear, reconstruction of auricular cartilage structure and its maintenance is very important. Use of biodegradable scaffolds has shortcomings in the sense that they show severe structural deformity during in vivo incubation [10]. In a recently published report, a nondegradable human ear-shaped implant was surface modified and subcutaneously implanted in nude mice. Although the scaffold was not deformed and maintained the original structure for 12 weeks of implantation, there were severe complications such as skin necrosis, implant exposure, and extrusion. When those scaffolds were covered with elastic chondrocytes from rabbit ear before implantation, there was no sign of such complications [27]. Consequently, an available source of elastic chondrocytes is essential for reconstruction of auricular cartilage of any kind.

A promising source of elastic chondrocytes has been found in auricular perichondrium. Previous studies have suggested that perichondrial tissue contains cells showing chondrogenic potential, and inner perichondrium-derived cells are particularly needed for cartilage regeneration [28, 29]. Cartilage stem/progenitor cells isolated from the auricular perichondrium have been characterized and shown to be capable of differentiating into adipocytes and osteocytes, suggesting that their differentiation potential is greater than that of predetermined chondrocytes. In addition, these cells exhibited CD44 and CD90 expression similar to that of mesenchymal stem cells (MSCs) and were called “ear-derived cartilage progenitor cells” (eCPCs) [30]. Moreover, subcutaneously transplanted eCPCs expanded in vitro were shown to regenerate elastic cartilage. However, when these cells were differentiated in vitro and transplanted onto a full-thickness cartilage defect of the joint, they regenerated hyaline cartilage tissue positive for type II and type X collagen and negative for type I collagen and elastin [31]. In contrast to these reports, our results showed that transplanting XCs onto a full-thickness defect of joint cartilage resulted in elastic cartilage tissue in approximately 34% of cases (Fig. 6; supplemental online Fig. 3). The different outcomes produced by eCPCs and XCs clearly indicate the differences in the differentiation potential of the respective cell types. As predetermined chondrocytes, XCs showed a reliable tendency to form elastic cartilage—an advantageous trait for autologous cell-derived elastic cartilage reconstruction.

Although cells from the perichondrium showed superior in vitro expansion ability, they exhibited drastic dedifferentiation after passage 3, suggesting inconsistency in the reproducibility of cartilage reconstruction and the rate of new cartilage formation [32]. It has been argued previously that the characteristics of cartilage stem/progenitor cells are unstable, based on the fact that their characteristics are affected by age, culture condition, and passage number [10, 32]. Considered in this context, the fact that chondrocytes isolated from cartilage also showed expression of type II collagen and aggrecan until passage 3 indicates that cartilage might be a preferable source of chondrocytes. Moreover, in cases in which perichondrium is not available, an additional

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**Table 1. Incidence of elastic cartilage formation in an ACI experiment**

| Source of autologous chondrocytes used in ACI | Elastic cartilage | Hyaline cartilage |
|---------------------------------------------|------------------|------------------|
| Xiphoid process                             | 9 of 26 knees (34.62%) | 17 of 26 knees (65.38%) |
| Costal cartilage                            | 0 of 12 knees (0%) | 12 of 12 knees (100%) |

Abbreviation: ACI, autologous chondrocyte implantation.

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**Figure 6.** CFU-F assay of rabbit XCs and in vivo chondrogenesis in the rabbit ACI model. (A): CFU-Fs of XCs were compared between rats and rabbits. Colonies consisting of >50 cells were counted. (B): The cartilage regeneration capacity of XCs and CCs was confirmed by autologously transplanting each type of cell in a gel-like state into a rabbit model of full-thickness articular cartilage defect. Representative H&E and Elastica van Gieson staining of normal articular cartilage, and XC- and CC-treated groups are shown. Cartilage regenerated by XCs in the ACI model exhibited an elastic cartilage morphology, as demonstrated by the presence of large lacunae and elastic fibers. The dotted circle shows elastic cartilage formation in the XC-transplanted group. Scale bar = 100 μm. Abbreviations: ACI, autologous chondrocyte implantation; CC, costal cartilage-derived chondrocyte; CFU-F, colony-forming unit fibroblast; H&E, hematoxylin and eosin; XC, xiphoid process-derived chondrocyte.
chondrocyte source of elastic cartilage is needed for elastic cartilage reconstruction.

A comparison of cell yields and CFU-Fs in XCs and CCs isolated from young and adult rats showed that the ratio of CFU-Fs was dramatically increased in XCs from aged animals, whereas the ratio of CFU-Fs in CCs was decreased. Considering the decreased cell yield in the aged animals, the actual number of CFU-Fs per milligram of tissue showed a tendency to decline with age. The numbers of CFU-Fs per milligram of tissue yielded by XCs from young and adult rats were 672.0 and 548.8, respectively—a decrease of approximately 18.3% with age. In contrast, the number of CFU-Fs per milligram of tissue for CCs decreased by more than 77% in old rats (67.4) compared with young rats (294.6). Collectively, these results demonstrate that chondrocyte precursors with colony-forming ability are maintained in xiphoid processes, even in old age.

A previous report showed that CCs exhibited superior initial cell yield, cell expansion, and differentiation potential compared with ACs [33], suggesting that CCs would be a better chondrocyte source for autologous chondrocyte implant. In the current study, we compared XCs with CCs, demonstrating that the characteristics of XCs are superior to those of CCs in terms of initial cell yield, in vitro expansion, and GAG production.

Previously, Puelacher et al. [34] tested seeding of varying densities of chondrocytes on a biocompatible polymer and suggested that a cell density of $2 \times 10^5$ to $10^6$ cells per cm$^3$ is needed for the growth of new cartilage tissue. Similarly, Shieh et al. [10] reported construction of auricular cartilage tissue by seeding adult sheep chondrocytes at a density of $5 \times 10^7$ cells per cm$^3$. Considering that $\sim 6 \times 10^6$ cells are obtained from 50 mg of tissue, chondrocytes could be expanded to approximately $8 \times 10^7$ cells at passage 3 and $1.3 \times 10^8$ cells at passage 5. If elastic cartilage tissue were constructed by seeding XCs at a density of $5 \times 10^7$ cells per cm$^3$, in vitro-expanded chondrocytes could cover approximately 16 cm$^3$ at passage 3 and 27 cm$^3$ at passage 5.

The high CFU-F-forming ability of XCs led us to examine the differentiation potential of these cells to determine whether they show characteristics of MSCs. In vitro differentiation potentials of rat XCs were compared with those of rat BMSCs, the gold standard for stem cells of the mesenchymal lineage. Compared with BMSCs, XCs showed lower adipogenic potential and no in vitro osteogenesis potential (data not shown) and exhibited superb chondrogenic potential in vitro and in vivo (Fig. 2). Considering that in vivo transplantation is intended to induce ectopic bone formation by attaching cells to HA/TCP granules, the demonstration of in vivo chondrogenesis by XCs in a highly osteogenic environment means that XCs retained the characteristics of their original tissue, even after in vitro expansion over four passages.

Yokoyama et al. have shown that auricular cartilage-derived chondrocytes produce more cartilaginous matrix when cultured with basic fibroblast growth factor and dexamethasone [2]. A comparison of matrix production by elastic chondrocytes and hyaline chondrocytes revealed significantly greater matrix formation by elastic chondrocytes in our experiments, ACs, CCs, and XCs cultured beyond confluence showed spontaneous chondrogenic ECM production, even without chondrogenic induction medium (supplemental online Fig. 2). Based on Alcian blue staining intensity, XCs showed the most abundant GAG production, an observation in agreement with the previous report by Yokoyama et al. [2].

When XCs were cultured beyond confluence, they formed a gel-like state, as shown in Figure 5A. Subcutaneous transplantation of XCs in this gel-like state into nude mice resulted in the production of cartilage tissue exhibiting large lacunae and scarce amounts of matrix in the interterritorial zone, features similar to those of elastic cartilage.

The presence of multipotent stem/progenitor cells in the perichondrium of the xiphoid process was not examined in this study. Examination of the presence of chondrogenic progenitor cells in the perichondrium, together with confirmation of the characteristic of human xiphoid process cartilage, is needed for translation to clinical research.

CONCLUSION

Xiphoid process cartilage is composed of hyaline cartilage at the proximal part of the xiphoid process and elastic cartilage at the distal part. Chondrocytes isolated from the elastic cartilage portion of the xiphoid process showed superb in vitro expansion ability and a highly chondrogenic nature. In vivo transplantation of XCs produced a typical elastic cartilage morphology. Collectively, these results suggest that the xiphoid process is a promising source of chondrocytes for reconstruction of elastic cartilage.

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AUTHOR CONTRIBUTIONS

S.N.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; W.C. and H.C.: collection and assembly of data; J.L.: conception and design; E.L.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Y.S.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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