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Improving In Vitro Cartilage Generation by Co-Culturing Adipose-Derived Stem Cells and Chondrocytes on an Allograft Adipose Matrix Framework

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Background: Microtia is an inherited condition that results in varying degrees of external ear deformities; the most extreme form is anotia. Effective surgical reconstruction techniques have been developed. However, these usually require multistage procedures and have other inherent disadvantages. Tissue engineering technologies offer new approaches in the field of external ear reconstruction. In this setting, chondrocytes are cultured in the laboratory with the aim of creating bioengineered cartilage matrices. However, cartilage engineering has many challenges, including difficulty in culturing sufficient chondrocytes. To overcome these hurdles, the authors propose a novel model of cartilage engineering that involves co-culturing chondrocytes and adipose-derived stem cells on an allograft adipose-derived extracellular matrix scaffold.
Methods: Auricular chondrocytes from porcine ear were characterized. Adipose-derived stem cells were isolated and expanded from human lipoaspirate. Then, the auricular chondrocytes were cultured on the allograft adipose matrix either alone or with the adipose-derived stem cells at different ratios and examined histologically.
Results: Cartilage induction was most prominent when the cells were co-cultured on the allograft adipose matrix at a ratio of 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio). Furthermore, because of the xenogeneic nature of the experiment, the authors were able to determine that the adipose-derived stem cells contributed to chondrogenesis by means of a paracrine stimulation of the chondrocytes.
Conclusions: In this situation, adipose-derived stem cells provide sufficient support to induce the formation of cartilage when the number of auricular chondrocytes available is limited. This novel model of cartilage engineering provides a setting for using the patient's own chondrocytes and adipose tissue to create a customized ear framework that could be further used for surgical reconstruction. (*Plast. Reconstr. Surg.* 147: 87, 2021.)

Microtia is a congenital condition that includes a spectrum of malformations of the external ear, ranging from minimal changes to the complete absence of the ear, or anotia.[1] Microtia occurs in one of every 4000 to 10,000 births, significantly impacting the patient and family emotionally and psychologically.[2] Plastic reconstructive surgical techniques

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to repair microtia typically include autogenous cartilage reconstruction, alloplastic implants, or prosthetic reconstruction.[3] Ear reconstruction is a challenging undertaking, because the normal external ear has a complex three-dimensional structure. The current widely accepted techniques by Brent and Nagata use autologous rib cartilage to create the external ear framework.[4,5] However, these approaches are associated with significant donor-site morbidity and an extensive learning curve for the surgeon.[6] Thus, there is a necessity for novel reconstructive approaches to treat this malformation.

Tissue engineering and regenerative medicine provide promising approaches in the field of external ear reconstruction, and autologous engineered tissue for defect repair and cartilage reconstruction have been demonstrated.[7,8] However, cartilage engineering is challenging. For example, chondrocytes are difficult to culture in vitro, because long-term culture and passaging of chondrocytes results in reduced collagen production and potential dedifferentiation or the loss of chondrocyte phenotypes.[9] In addition, in vitro–generated cartilage shows an inferior structure and function compared to native cartilage.[10] To overcome these complications, cartilage engineering research has used two main approaches, including the use of co-cultures to expand the number of chondrogenic cells and culturing on three-dimensional scaffolds that resemble the native environment of chondrocytes. Co-culturing mesenchymal stem cells with chondrocytes is an effective way to increase chondrocyte vitality and decrease dedifferentiation.[11] There are two main hypotheses explaining the efficacy of this approach. The first states that mesenchymal stem cells directly differentiate into chondrocytes, and the second states that mesenchymal stem cells release paracrine factors that promote chondrocyte growth.[12,13]

Furthermore, direct cell-to-cell contact between chondrocytes and mesenchymal stem cells is an important factor for co-culture success.[12] When human nasal chondrocytes and human bone marrow–derived stem cells were co-cultured on a three-dimensionally–bioprinted hydrogel scaffold and implanted in mice, the degree of chondrogenesis and chondrocyte proliferation was better than the explants with chondrocytes alone,[14] highlighting the additive effect of mesenchymal stem cells. However, the use of bone marrow–derived stem cells for translational purposes requires an invasive and painful harvesting procedure. In the clinical setting, adipose-derived stem cells, a particular subset of mesenchymal stem cells, are feasible because they are abundant in fat tissue and are easily harvested, with minimal donor-site morbidity. In addition, the aforementioned study used a synthetic three-dimensionally–bioprinted hydrogel. Adipose tissue is a better alternative in this clinical scenario, not only as a cell source, but also because it is a biological scaffold for the initial cell support for chondrogenesis.[15] The concept of allograft adipose matrix has emerged in the literature, and our laboratory explored variations in its preparation and design.[16] Thus, we hypothesized that allograft adipose matrix, a native extracellular matrix, serves as a framework for co-culturing adipose-derived stem cells and chondrocytes to induce chondrogenesis. Ultimately, this study will provide a novel model of cartilage engineering for ear reconstruction.

MATERIALS AND METHODS

Porcine Auricular Chondrocyte Isolation

Auricular chondrocytes were isolated from the external ears of male Yorkshire pigs (2 to 3 weeks old) (tissue was from killed pigs; therefore, this study was exempt from institutional animal care and use committee approval). The cartilage was separated and washed in sterile phosphate-buffered saline with 1% penicillin and streptomycin (Corning Cellgro; Thomas Scientific, Swedesboro, N.J.). The cartilage was cut and was digested in collagenase II (Gibco, Gaithersburg, Md.). The collagenase was neutralized with 10% fetal bovine serum (Fisher Scientific, Waltham, Mass.). The digested solution was filtered over a 100-μm nylon mesh (Fisher), and the cell suspension was centrifuged. The cell pellet was resuspended in commercially available chondrocyte growth medium (PromoCell, St. Louis, Mo.).

Adipose-Derived Stem Cell Isolation, Expansion, and Characterization

Fat tissue was obtained from healthy individuals undergoing elective liposuction at the Department of Plastic Surgery, University of California, Irvine (institutional review board no. 2015-2181). The lipoaspirate was washed in phosphate-buffered saline, digested in collagenase I (Sigma, St. Louis, Mo.), and neutralized using Dulbecco's Modified Eagle Medium (Corning Cellgro) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Then, the cells were centrifuged and the pellet was resuspended in Dulbecco's Modified Eagle Medium

plus 10% fetal bovine serum plus 1% penicillin/streptomycin. The isolated adipose-derived stem cells were characterized as described previously by our laboratory.[17]

Glycosaminoglycan Staining

Auricular chondrocytes, at the indicated passages, were grown to confluence, washed, fixed in 10% formalin (EMD Millipore Corp., Burlington, Mass.), and stained with Alcian blue (Sigma). Washed cells were imaged directly using a phase-contrast microscope (Olympus IX70; Olympus Corp., Tokyo, Japan). Three images were obtained per dish, and representative images are presented. This experiment was repeated three times.

Immunofluorescent Type II Collagen Staining

Auricular chondrocytes, at the indicated passages, were grown to confluence in four-well chamber slides (Labtek, Scotts Valley, Calif.) and were washed, fixed in paraformaldehyde (Fisher), permeabilized, blocked in bovine serum albumin (Sigma), and incubated with a type II collagen primary antibody (Abcam, Cambridge, United Kingdom). After washing, the cells were incubated with a goat anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, Calif.). The cells were imaged using an Evos fluorescent microscope (Thermo Fisher Scientific, Waltham, Mass.). Three fields of view were imaged per chamber, and the fluorescent images were analyzed using ImageJ (National Institutes of Health, Bethesda, Md.). The experiment was repeated three times.

Reverse-Transcriptase Polymerase Chain Reaction

Auricular chondrocytes and adipose-derived stem cells were cultured either alone or were cultured at a ratio of 1:5 or 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio). After 48 hours, RNA was isolated using Trizol (Invitrogen) following the manufacturer's recommendations. The RNA was then reverse transcribed using the SuperScript IV One-Step RT-PCR System (Invitrogen), which included a DNase treatment. The primers used for the amplification were as follows:

1. Collagen II

   Forward: 5′-GAGGTCTTCCTGGCAAAGAT-3′
   Reverse: 5′-GTCCCTGGAAGCCAGATG-3′

2. SOX9

   Forward: 5′-TCGCAGTACGACTACACAGA-3′
   Reverse:5′-TGTAGGTGAAGGTGGAGTAGAG-3′

3. β-actin

   Forward: 5′-CTTCCAGCAGATGTGGATCAG-3′
   Reverse: 5′-CAGTCCGCCTAGAAGCATTT-3′

The primers were synthesized by Integrated DNA Technologies (Coralville, Iowa), and β-actin was used as the housekeeping gene for normalization. The cDNA amplification was detected using gel electrophoresis and the bands were quantified using ImageJ. The experiment was repeated twice.

Allograft Adipose Matrix Framework Preparation

The allograft adipose matrix framework was obtained from human cadaveric donor adipose tissue provided by the Musculoskeletal Transplant Foundation (Edison, N.J.). The donor tissue was screened for pathogens. The adipose tissue was separated from the subcutaneous layer of full-thickness skin and mechanically reduced and centrifuged to isolate the adipose fraction. The chemical processing steps involved the use of an organic solvent to remove the lipid component and a surfactant/ethanol-based solution to remove the cellular content and debris, while preserving the integrity of the extracellular matrix. The matrix was disinfected to ensure tissue safety. Finally, the matrix was lyophilized in a 24-well tissue culture plate, and the resulting disks were trimmed and cut to size.

Culturing Cells on the Allograft Adipose Matrix Framework

The disk-shaped allograft adipose matrix framework was approximately 15 mm in diameter and was approximately 1 to 2 mm thick. For the co-culture, the adipose-derived stem cells and auricular chondrocytes were seeded either alone or together on the allograft adipose matrix in chondrocyte medium (PromoCell), and the total number of cells per framework was 6 million. The auricular chondrocyte–to–adipose-derived stem cell ratios were 1:5 and 1:9, and auricular chondrocytes and adipose-derived stem cells alone served as controls. All constructs were cultured for 10 weeks.

Hematoxylin and Eosin Staining and Immunohistochemical Evaluation

After culturing, the allograft adipose matrix framework was fixed in paraformaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin. Representative images are presented. For the immunohistochemical analysis, the expression of type II collagen was detected using a rabbit

anti-human type II collagen antibody (Abcam) followed by a horseradish peroxidase–conjugated anti-rabbit antibody (Abcam) and color development with diaminobenzidine tetrahydrochloride (Abcam) as described previously.[18]

Enzyme-Linked Immunosorbent Assay

After the allograft adipose matrix disks were cultured as described above, samples from the different groups (n = 3) were homogenized, and the supernatants were used to quantify porcine hydroxy lysyl pyridinoline using an enzyme-linked immunosorbent assay kit (MyBioSource, San Diego, Calif.), according to the manufacturer's instructions. The data were normalized to the human-derived adipose-derived stem cells, which were set to zero, because they should not be positive for porcine hydroxy lysyl pyridinoline. The experiment was conducted in triplicate and repeated twice.

The allograft adipose matrix disks were cultured as described above, and the supernatants were collected weekly. Enzyme-linked immunosorbent assay kits (MyBioSource) for human and porcine type II collagen were used following the manufacturer's instructions. The human collagen II results were normalized to the chondrocytes because they were presumed to be negative, and the porcine collagen II results were normalized to the adipose-derived stem cells, which were presumed to be negative. The enzyme-linked immunosorbent assays were conducted using the supernatants that were collected at weeks 3, 5, 7, and 9. The experiment was conducted in triplicate and repeated twice.

Statistical Analysis

All the quantitative data are expressed as the mean ± SD. A t test was performed to assess the differences between the groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Chondrocyte Isolation and Assessment

First, we determined the best method for expanding and storing the isolated auricular chondrocytes. This was a crucial step because the experiment required a large quantity of chondrocytes, which could only be achieved by expanding the cells after the primary isolation. The main concern with expanding the cells is the potential for chondrocyte dedifferentiation.[9] We used two sets of auricular chondrocytes from the same initial porcine ear isolation. The first set was a freshly isolated and passaged set of cells. The cells used were from the initial isolation, the first passage, the second passage, and the third passage, which were derived from freshly isolated cell lines maintained in culture without freezing. The second set of cells was taken from the initial isolation cells mentioned above, which were frozen in liquid nitrogen and were later thawed for the experiments, which meant that these were used at passage 1 (P1_frozen). Then, the passage 1 cells described above were also frozen in liquid nitrogen and were later thawed and used at passage 2 (P2_frozen). Finally, the passage 2 cells described above were also frozen in liquid nitrogen and were later thawed and used at passage 3 (P3_frozen).

Alcian blue staining was used to assess the glycosaminoglycan content. All of the passages expressed glycosaminoglycans, but between passage 2 (Fig. 1, *above*) and passage 3 (Fig. 1, *below*), for both the freshly isolated cells (Fig. 1, *left*) and the frozen cells (Fig. 1, *right*), there was a decline in the ability of cells to achieve confluence. We also used fixed and unstained chondrocytes as a negative control (data not shown).

Next, the freshly isolated passage 0 (Fig. 2, *above*, *left*), passage 1 (Fig. 2, *above*, *right*), passage 2 (Fig. 2 *second row*, *left*), and passage 3 (Fig. 2, *second row*, *right*) auricular chondrocytes were grown to confluence and were stained for collagen type II using immunofluorescence. In addition, auricular chondrocytes from the same isolation were frozen, thawed, and expanded at passage 1 (Fig. 2, *third row*, *left*), passage 2 (Fig. 2, *third row*, *right*), and passage 3 (Fig. 2, *below*, *left*) and were stained in the same manner. The fluorescence intensity was quantified for each of the passages (Fig. 2, *below*, *right*). For the negative control, the primary antibody was omitted from the staining procedure (data not shown). The type II collagen staining decreased with passage number and was more pronounced when the auricular chondrocytes were frozen and thawed. Thus, for all of the future experiments, the isolated auricular chondrocytes were frozen at passage 0, and for the experiments, the initial isolation cells were expanded (passage 1) and were seeded on the allograft adipose matrix at passage 2.

Assessing Gene Expression after Co-Culturing Auricular Chondrocytes and Adipose-Derived Stem Cells

Next, we examined the gene expression of type II collagen and *SOX9*. Auricular chondrocytes and adipose-derived stem cells were cultured either alone or together at ratios of 1:5 or 1:9 (auricular

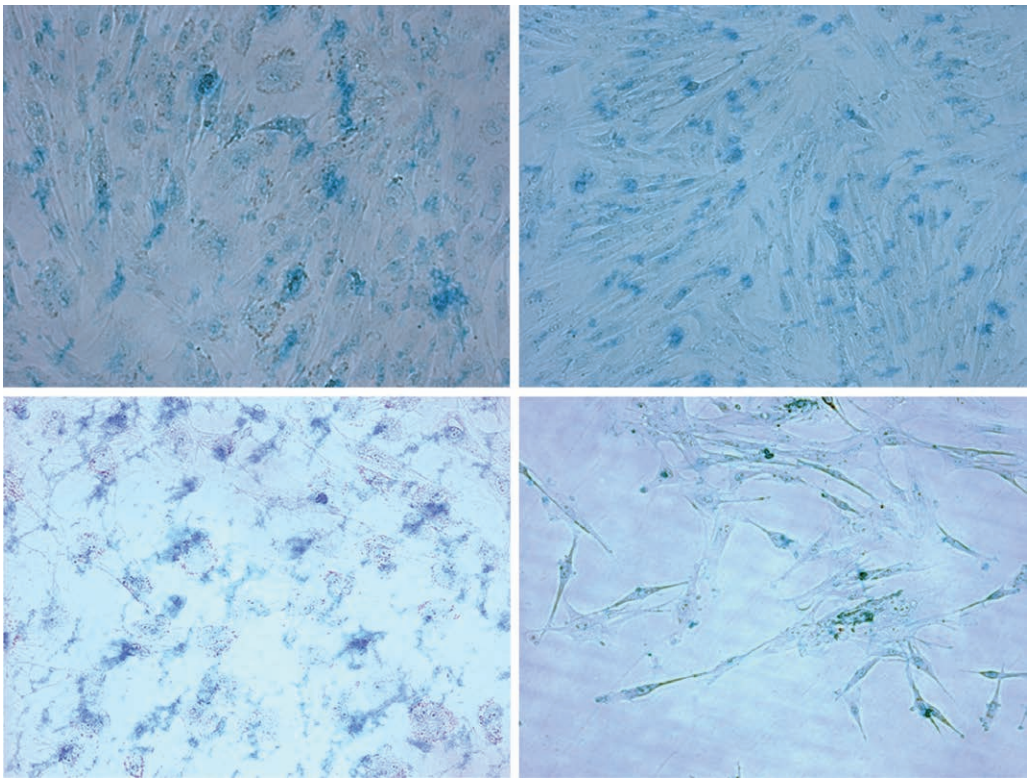

Fig. 1. Porcine auricular chondrocytes express glycosaminoglycan in vitro. (*Above*, *left*) Freshly isolated auricular chondrocytes at passage 2. (*Above*, *right*) Frozen and thawed auricular chondrocytes at passage 2. (*Below*, *left*) Freshly isolated auricular chondrocytes at passage 3. (*Below*, *right*) Frozen and thawed auricular chondrocytes at passage 3 (original magnification, × 4). Representative images are presented.

chondrocyte–to–adipose-derived stem cell ratio). The type II collagen expression was significantly increased when the cells were cultured together in comparison to the auricular chondrocytes and adipose-derived stem cells alone, and the expression at the ratio of 1:9 was significantly greater than the 1:5 ratio (Fig. 3, *above*). *SOX9* expression was significantly increased when cells were cultured together at both ratios in comparison to the auricular chondrocytes alone (Fig. 3, *below*). Together, these data set the foundation that the co-culture conditions increased the expression of genes related to chondrogenesis compared to culturing the auricular chondrocytes alone.

Adipose-Derived Stem Cell and Auricular Chondrocyte Co-Culture on the Allograft Adipose Matrix Framework

Next, after 10 weeks of culture, the hematoxylin and eosin staining framework revealed that each cell type was present when the adipose-derived stem cells and auricular chondrocytes were cultured alone on the allograft adipose matrix (Fig. 4, *above*, *left*, and *above*, *right*, respectively). However, when the cells were co-cultured at 1:5 and 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio), there was evidence of tissue changes (Fig. 4, *below*, *left*, and *below*, *right*, respectively). Both co-culture conditions revealed extracellular matrix deposition, but the 1:9 group showed a more compact organization, which was suggestive of cartilage formation (Fig. 4, *below*, *right*).

In addition, type II collagen staining was assessed by immunohistochemistry (Fig. 5). The antibody was specific for both human and porcine type II collagen and thus represented an overview of type II collagen expression from both cell types. The 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio) co-culture showed the highest level of type II collagen in the allograft adipose matrix framework, and this was significantly greater than the adipose-derived stem cells and chondrocytes alone. This experiment was repeated twice, with similar results.

Differentiating the Contribution of the Auricular Chondrocytes and Adipose-Derived Stem Cells Cultured on Allograft Adipose Matrix to Induce Chondrogenesis

Because of the xenogeneic nature of the experiment, we used species-specific antibody tests to examine the contribution of each cell

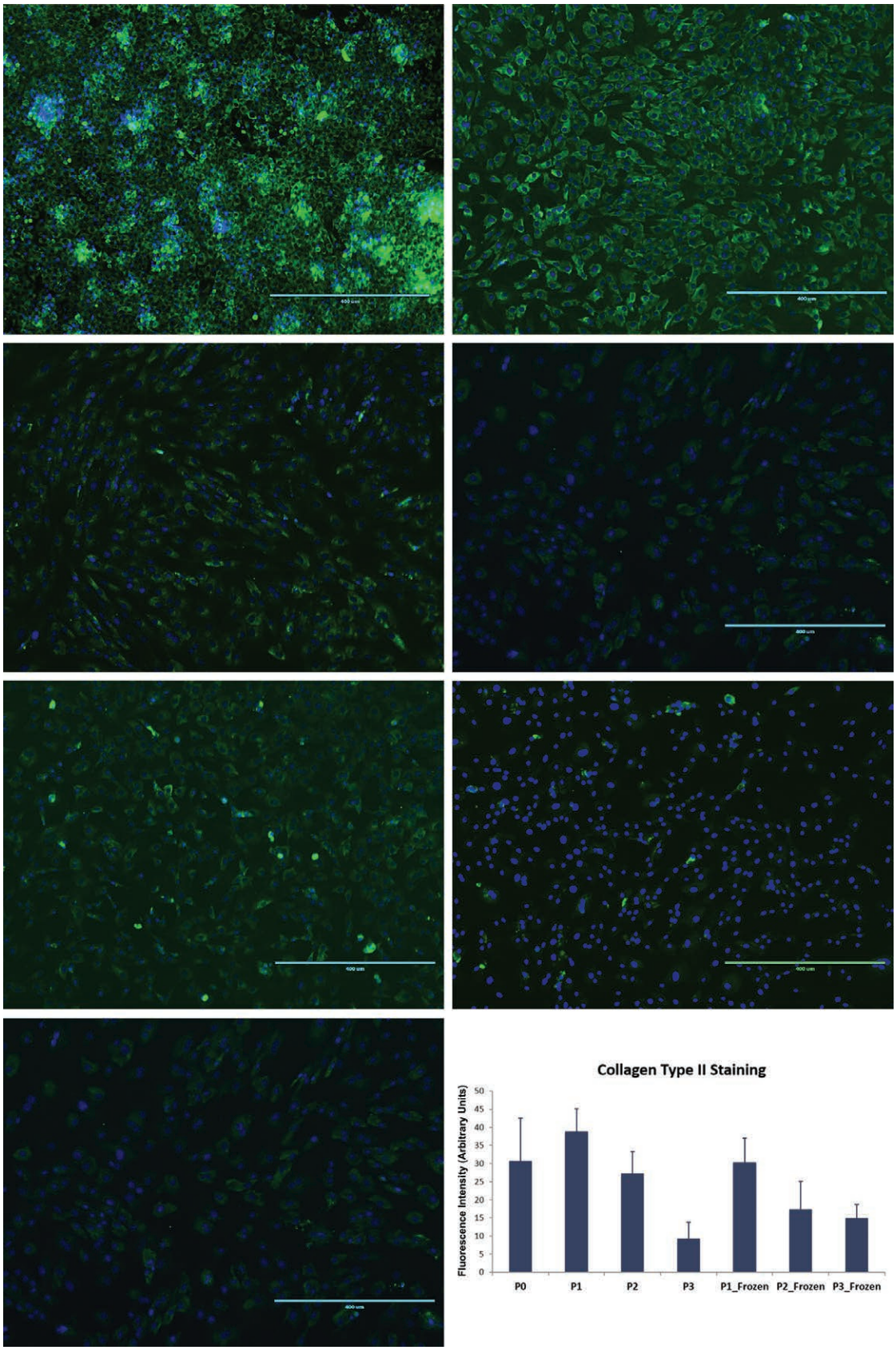

Fig. 2. Type II collagen expression in porcine auricular chondrocytes in vitro. Freshly isolated passage 0 (*above*, *left*), passage 1 (*above*, *right*), passage 2 (*second row*, *left*), and passage 3 (*second row*, *right*) auricular chondrocytes

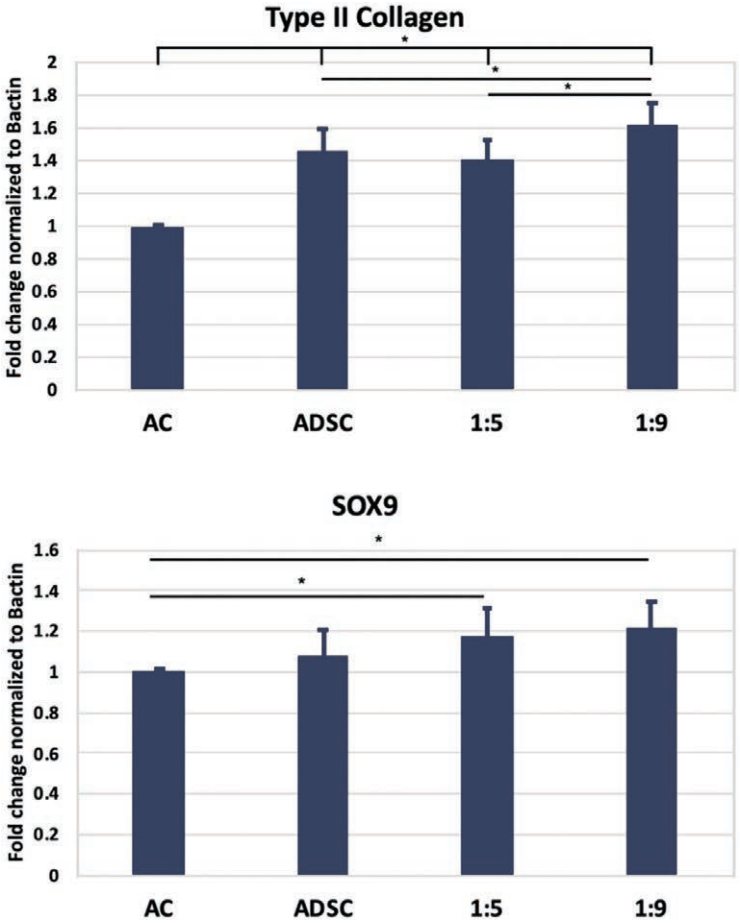

Fig. 3. Type II collagen and *SOX9* gene expression after co-culturing. Auricular chondrocytes and adipose-derived stem cell were cultured alone or together at ratios of 1:5 or 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio). After 48 hours, RNA was isolated and the gene expression of (*above*) type II collagen and (*below*) *SOX9* was determined by reverse-transcriptase polymerase chain reaction. The data were normalized to β-actin and are presented as the mean ± SD of two independent experiments (*$p < 0.05$).

type to chondrogenesis. First, we assessed the expression of porcine hydroxy lysyl pyridinoline by enzyme-linked immunosorbent assay to determine whether there was evidence of collagen crosslinking. Because the auricular chondrocytes were porcine derived and the adipose-derived stem cells were human, the adipose-derived stem cells served as a negative control, and the data were normalized based on the baseline level of the adipose-derived stem cells. Indeed, the auricular chondrocytes alone showed hydroxy lysyl pyridinoline expression when cultured on allograft adipose matrix, and the addition of adipose-derived stem cells increased that expression at the 1:9 ratio but not at the 1:5 ratio. In addition, the increase in hydroxy lysyl pyridinoline expression between the 1:5 and 1:9 co-cultures was statistically significant (Fig. 6). These data revealed that co-culturing auricular chondrocytes and adipose-derived

Fig. 2. (*Continued*). were grown to confluence and were stained for collagen type II using immunofluorescence. In addition, auricular chondrocytes from the same isolation were frozen, thawed, and expanded at passage 1 (*third row*, *left*), passage 2 (*third row*, *right*), and passage 3 (*below*, *left*) and were stained in the same manner. The images are a merge of collagen type II staining in green and 4′,6-diamidino-2-phenylindole in blue. The images were acquired at 10× using the same microscope parameters, and (*below*, *right*) the fluorescence intensity was determined using ImageJ with the same settings for each image. The data are presented as the mean ± SD of three independent experiments.

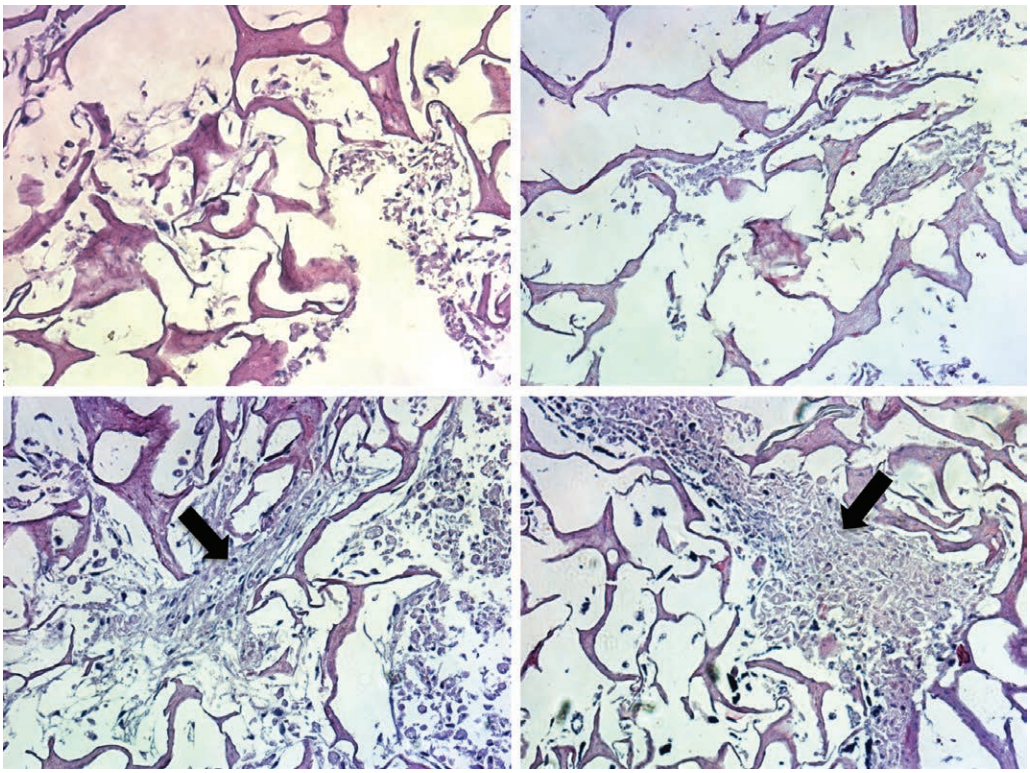

Fig. 4. Co-culturing adipose-derived stem cells and auricular chondrocytes on the allograft adipose matrix framework. The adipose-derived stem cells and auricular chondrocytes were seeded onto the framework alone or in combination and were cultured for 10 weeks. The total number of cells for each condition was 6 million. The disks were then fixed and stained with hematoxylin and eosin. (*Above*, *left*) Adipose-derived stem cells alone. (*Above*, *right*) Auricular chondrocytes alone. (*Below*, *left*) Auricular chondrocyte–to–adipose-derived stem cell ratio of 1:5. (*Below*, *right*) Auricular chondrocyte–to–adipose-derived stem cell ratio of 1:9 (original magnification, × 10). The *arrows* represent areas of extracellular matrix deposition. Representative images are presented.

stem cells at a ratio of 1:9 increased the collagen crosslinking compared to the auricular chondrocytes alone, demonstrating the trophic effect of the adipose-derived stem cells.

Finally, we examined the species-specific secretion of type II collagen using enzyme-linked immunosorbent assay. The assessment of human collagen II secretion reflected the collagen II secretion by the adipose-derived stem cells, revealing that there was essentially no human collagen II secretion throughout the experiment (Fig. 7, *above*). However, for the porcine secretion, which is a reflection of the collagen II secreted by the auricular chondrocytes, there was a time-dependent increase for the 1:9 co-culture condition, with the 9-week time point showing a significant increase compared to the 3-week time point. The other conditions did not reveal a distinct pattern, indicating that the 1:5 co-culture was not different from culturing the chondrocytes alone (Fig. 7, *below*). Together, these data demonstrated that the increased collagen II was auricular chondrocyte–derived and that the increase was attributable to the addition of adipose-derived stem cells at a ratio of 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio).

DISCUSSION

Autologous cartilage reconstruction is the standard treatment modality for microtia, and the Brent and Nagata techniques, along with the use of soft-tissue expanders, are mainly used.[19] However, complications arise with these techniques.[20] The notion of generating an ear-shaped cartilage framework by means of tissue engineering is not new.[21,22] Nevertheless, cartilage is a unique tissue that is difficult to create in a laboratory setting.[23] Two unmet needs in the field of cartilage engineering include increasing the number of chondrogenic cells[12,13,15,23–26] and identifying the appropriate scaffold to provide the

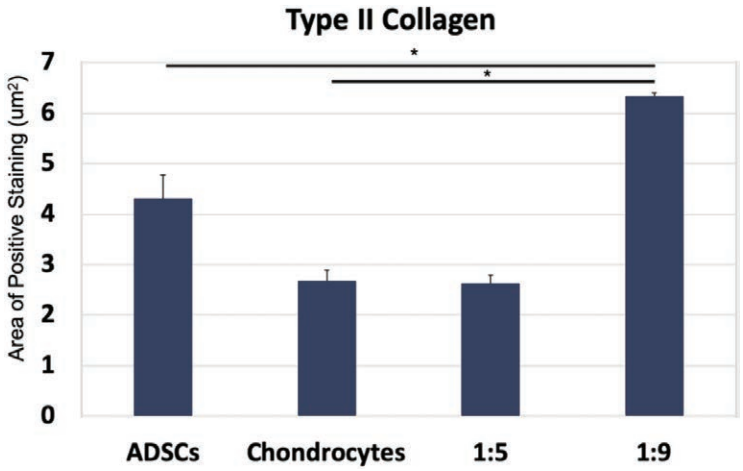

Fig. 5. Type II collagen immunohistochemical staining. After 10 weeks of co-culture, the allograft adipose matrix disks were harvested, fixed, and embedded in paraffin. Immunohistochemistry was performed to assess the type II collagen staining. The antibody detected both human and porcine type II collagen. The area of positive staining was assessed using ImageJ. The data are presented as the mean ± SD of two independent experiments (*$p < 0.05$).

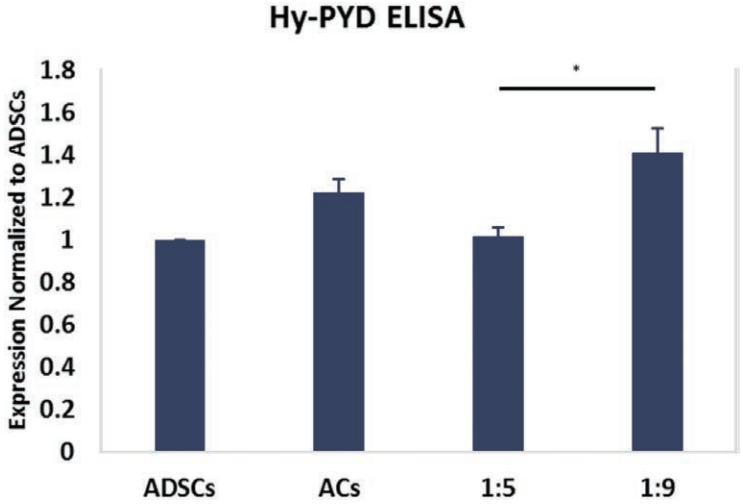

Fig. 6. Porcine hydroxy lysyl pyridinoline enzyme-linked immunosorbent assay. The allograft adipose matrix disks that were harvested for hematoxylin and eosin staining were also collected for enzyme-linked immunosorbent assay to assess the porcine hydroxy lysyl pyridinoline levels. The data were normalized to the adipose-derived stem cells, which were presumed to be negative for hydroxy lysyl pyridinoline expression. The data are presented as the mean ± SD of two independent experiments (*$p < 0.05$).

three-dimensional environment necessary for the initial stages of chondrogenesis.[12,15,23,27–29] Allograft adipose matrix, a novel biological material, preserves the essential components of native extracellular matrix.[16,30] Thus, we hypothesized that allograft adipose matrix, despite its adipose origin, provides the adequate critical attachment sites for chondrogenic cells, and co-culturing auricular chondrocytes and adipose-derived stem cells on allograft adipose matrix would generate a chondrogenic setting that was better than auricular chondrocytes alone.

As previously described,[9] there was a loss of collagen expression after the auricular chondrocytes

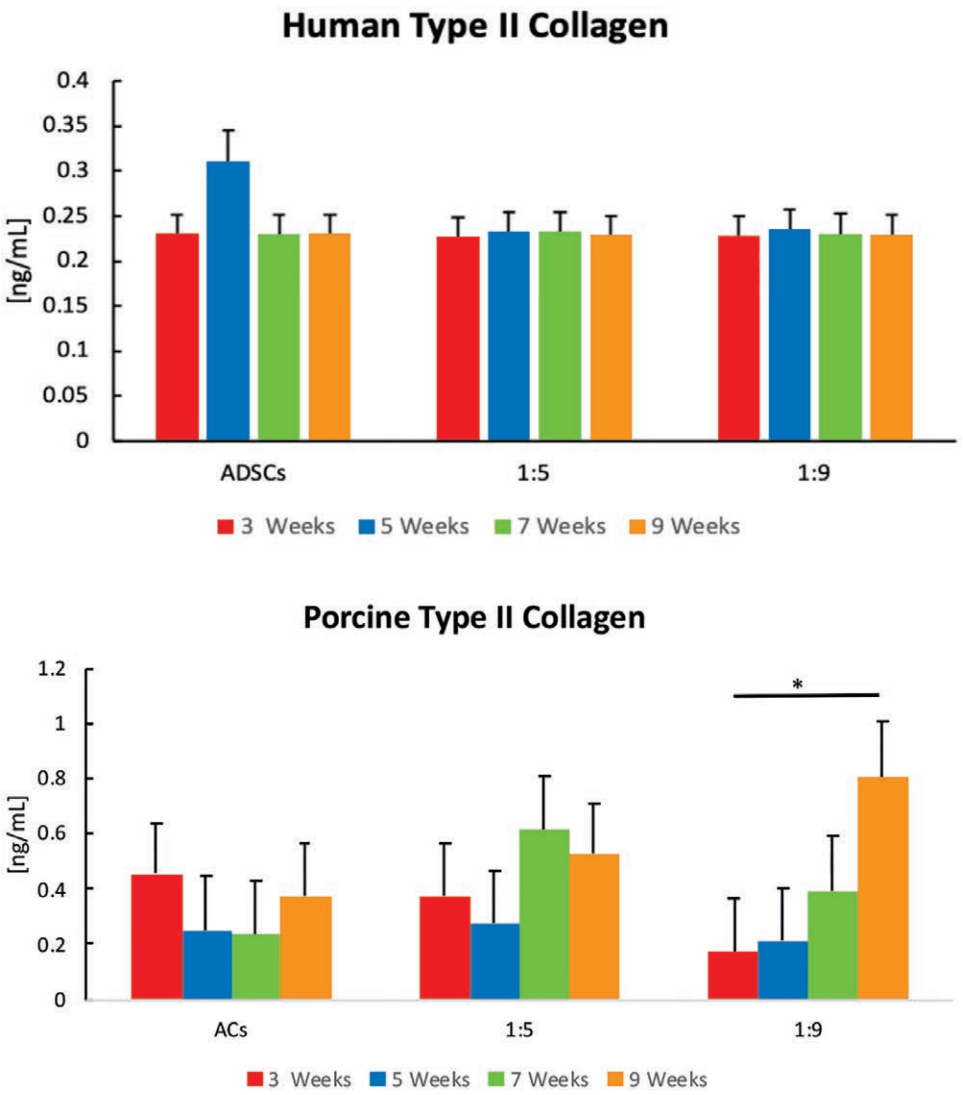

Fig. 7. Species-specific type II collagen expression. The allograft adipose matrix disks were cultured as described above, and each week the supernatants were collected. Enzyme-linked immunosorbent assay kits for human and porcine type II collagen were used to assess the levels in the samples. (*Above*) The human collagen II results were normalized to the chondrocytes because they were presumed to show no expression, and (*below*) the porcine collagen II results were normalized to the adipose-derived stem cells, which were presumed to show no expression. The enzyme-linked immunosorbent assays were conducted using the supernatants from the collections at weeks 3, 5, 7, and 9. The data are presented as the mean ± SD of two independent experiments (*$p < 0.05$).

were passaged multiple times. Furthermore, confluent cell growth was not observed as the passage number increased. These results supported the notion that chondrocytes are difficult to culture long term. When chondrocytes are cultured in a monolayer and dedifferentiate in the setting of cartilage engineering, the resulting cartilage is usually fibrous and mechanically inferior.[31] Thus, co-culture approaches have emerged for generating cartilage in vitro and in vivo.[13,25,26] To establish that co-culturing auricular chondrocytes and adipose-derived stem cells was indeed chondroinductive, we examined the gene expression of type II collagen and *SOX9*, which are key regulators of chondrogenesis.[32] These data provided evidence that the induction of type II collagen and *SOX9* occurred to a greater degree in the adipose-derived stem cells alone compared to the auricular chondrocytes alone. However, when the two cells were co-cultured, the induction of these genes was significantly increased compared to the cells alone. The lower expression of these genes

in the auricular chondrocytes contributes to the notion that auricular chondrocytes lose their chondrogenic potential when they are grown in culture.[9] In addition, *SOX9* contributes to the differentiation of adipose-derived stem cells into a chondrocyte-like phenotype in vitro.[33] The adipose-derived stem cells here were grown in chondrogenic medium, which may provide the cues for pushing the cells toward a more chondrocyte-like gene expression profile. This is an interesting observation; however, to provide a better model for chondrogenesis, we used auricular chondrocytes and adipose-derived stem cells, with allograft adipose matrix as the scaffold. The allograft adipose matrix used here was derived from cadaveric donor tissues using a standardized and controlled method, which offers the opportunity for large-scale manufacturing to create an off-the-shelf scaffold. The generation of human cadaver-derived acellular scaffolds from other tissues has previously shown the high value of this approach.[34,35] Here, the 1:9 culture (auricular chondrocyte–to–adipose-derived stem cell ratio) provided the best outcome for chondrogenesis. Indeed, when the chondrocyte number in a co-culture with mesenchymal stem cells is reduced, chondrocyte expansion is no longer necessary, allowing for the use of freshly isolated primary chondrocytes, which leads to improved cartilage formation.[36]

Cartilage formation is not the consequence of the chondrogenic lineage differentiation of mesenchymal stem cells,[37–40] and our data support this notion. In addition, newly formed cartilage matrix clearly originates from the chondrocytes, suggesting that the mesenchymal stem cells (both bone marrow–derived stem cells and adipose-derived stem cells) play a predominantly trophic role.[40] Here, the xenogeneic co-culture system permitted us to draw conclusions about the cell type responsible for chondrogenesis. By examining hydroxy lysyl pyridinoline and type II collagen secretion, we confirmed that the auricular chondrocytes were indeed responsible for chondroinduction and that the addition of adipose-derived stem cells at a ratio of 1:9 (auricular chondrocyte–to–adipose-derived stem cell ratio) supported this process. Previous studies also demonstrate that chondrocytes show an enhanced cartilage-forming capacity and proliferation activity in the presence of mesenchymal stem cells.[13,41–43] The allograft adipose matrix provided a framework for chondroinduction when auricular chondrocytes and adipose-derived stem cells were co-cultured, and the findings support the hypothesis that the adipose-derived stem cells function in a paracrine manner.

Three-dimensional bioprinting has also been used to create cartilage and even ear-shaped cartilage.[44,45] Zhou et al. used autologous chondrocytes derived from microtia cartilage. Auricular chondrocytes were seeded onto a synthetic ear-shaped scaffold, creating an in vitro system for generating cartilage that was implanted into humans.[45] However, this study was limited by exclusively using chondrocytes, and it is clear that culturing chondrocytes alone results in a limited capacity for chondrogenesis in vitro.[9] In addition, the biomaterials used for the scaffold were synthetic and required degradation in vivo after implantation. The inner core of the scaffold remained 1.5 years after implantation. Based on our findings, allograft adipose matrix, as a bioscaffold, provides an ideal chondroinductive platform for co-culturing chondrocytes and adipose-derived stem cells. Thus, we hypothesize that this model can also be used for three-dimensional printing to establish an ear-shaped cartilage that is more similar to the native ear and would be a novel treatment for microtia patients. Ultimately, a cartilage construct made in this manner may also solve issues related to the implantation process, including deformation challenges, because this framework may produce cartilage that possesses more native properties compared to other in vitro–generated cartilage constructs.

Our study has several limitations. First, the lyophilized bioscaffold did not provide pores for the cells to penetrate through the entire disk. Thus, the cells stayed on one side of the disk. In the future, by three-dimensionally printing the allograft adipose matrix, we will have better control over the porosity to establish a scaffold that is capable of being fully infiltrated by cells, improving chondrogenesis. Second, the adipose-derived stem cells were human derived and the auricular chondrocytes were porcine. Although this provided us with the opportunity to examine the contribution to chondrogenesis by cell type, this is not ideal the clinical model. In the future, we plan to perform these experiments using auricular chondrocytes from human cartilage. Ultimately, the goal is to obtain adipose-derived stem cells and cartilage from the same patient to perform autologous ear reconstruction with in vitro–generated cartilage.

CONCLUSIONS

The presence of adipose-derived stem cells supports the induction of cartilage when the number of auricular chondrocytes is limited, and allograft adipose matrix provides the proper framework for this process. This novel model of cartilage engineering

will provide a mode for using a patient's own chondrocytes and adipose tissue to create a customized ear framework that could be used for further surgical reconstruction to treat conditions such as microtia.

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REFERENCES

1. van Nunen DP, Kolodzynski MN, van den Boogaard MJ, Kon M, Breugem CC. Microtia in the Netherlands: Clinical characteristics and associated anomalies. *Int J Pediatr Otorhinolaryngol.* 2014;78:954–959.
2. Cabin JA, Bassiri-Tehrani M, Sclafani AP, Romo T III. Microtia reconstruction: Autologous rib and alloplast techniques. *Facial Plast Surg Clin North Am.* 2014;22:623–638.
3. Johns AL, Lucash RE, Im DD, Lewin SL. Pre and post-operative psychological functioning in younger and older children with microtia. *J Plast Reconstr Aesthet Surg.* 2015;68:492–497.
4. Brent B. Auricular repair with autogenous rib cartilage grafts: Two decades of experience with 600 cases. *Plast Reconstr Surg.* 1992;90:355–374; discussion 375–376.
5. Nagata S. A new method of total reconstruction of the auricle for microtia. *Plast Reconstr Surg.* 1993;92:187–201.
6. Baluch N, Nagata S, Park C, et al. Auricular reconstruction for microtia: A review of available methods. *Plast Surg (Oakv.)* 2014;22:39–43.
7. Kock L, van Donkelaar CC, Ito K. Tissue engineering of functional articular cartilage: The current status. *Cell Tissue Res.* 2012;347:613–627.
8. Raghunath J, Salacinski HJ, Sales KM, Butler PE, Seifalian AM. Advancing cartilage tissue engineering: The application of stem cell technology. *Curr Opin Biotechnol.* 2005;16:503–509.
9. Sykes JG, Kuiper JH, Richardson JB, Roberts S, Wright KT, Kuiper NJ. Impact of human platelet lysate on the expansion and chondrogenic capacity of cultured human chondrocytes for cartilage cell therapy. *Eur Cell Mater.* 2018;35:255–267.
10. van Osch GJ, Brittberg M, Dennis JE, et al. Cartilage repair: Past and future. Lessons for regenerative medicine. *J Cell Mol Med.* 2009;13:792–810.
11. Nazempour A, Van Wie BJ. Chondrocytes, mesenchymal stem cells, and their combination in articular cartilage regenerative medicine. *Ann Biomed Eng.* 2016;44:1325–1354.
12. de Windt TS, Saris DB, Slaper-Cortenbach IC, et al. Direct cell-cell contact with chondrocytes is a key mechanism in multipotent mesenchymal stromal cell-mediated chondrogenesis. *Tissue Eng Part A* 2015;21:2536–2547.
13. Wu L, Leijten JC, Georgi N, Post JN, van Blitterswijk CA, Karperien M. Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. *Tissue Eng Part A* 2011;17:1425–1436.
14. Apelgren P, Amoroso M, Lindahl A, et al. Chondrocytes and stem cells in 3D-bioprinted structures create human cartilage in vivo. *PLoS One* 2017;12:e0189428.
15. Choi JS, Kim BS, Kim JD, Choi YC, Lee HY, Cho YW. In vitro cartilage tissue engineering using adipose-derived extracellular matrix scaffolds seeded with adipose-derived stem cells. *Tissue Eng Part A* 2012;18:80–92.
16. Banyard DA, Borad V, Amezcua E, Wirth GA, Evans GR, Widgerow AD. Preparation, characterization, and clinical implications of human decellularized adipose tissue extracellular matrix (hDAM): A comprehensive review. *Aesthet Surg J.* 2016;36:349–357.
17. Banyard DA, Salibian AA, Widgerow AD, Evans GR. Implications for human adipose-derived stem cells in plastic surgery. *J Cell Mol Med.* 2015;19:21–30.
18. Liu K, Zhou GD, Liu W, et al. The dependence of in vivo stable ectopic chondrogenesis by human mesenchymal stem cells on chondrogenic differentiation in vitro. *Biomaterials* 2008;29:2183–2192.
19. Wilkes GH, Wong J, Guilfoyle R. Microtia reconstruction. *Plast Reconstr Surg.* 2014;134:464e–479e.
20. Fu YY, Li CL, Zhang JL, Zhang TY. Autologous cartilage microtia reconstruction: Complications and risk factors. *Int J Pediatr Otorhinolaryngol.* 2019;116:1–6.
21. Cao Y, Vacanti JP, Paige KT, Upton J, Vacanti CA. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. *Plast Reconstr Surg.* 1997;100:297–302; discussion 303–304.
22. Paige KT, Cima LG, Yaremchuk MJ, Schloo BL, Vacanti JP, Vacanti CA. De novo cartilage generation using calcium alginate-chondrocyte constructs. *Plast Reconstr Surg.* 1996;97:168–178; discussion 179–180.
23. Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noël D. Cartilage engineering: A crucial combination of cells, biomaterials and biofactors. *Trends Biotechnol.* 2009;27:307–314.
24. Baugé C, Boumédiene K. Use of adult stem cells for cartilage tissue engineering: Current status and future developments. *Stem Cells Int.* 2015;2015:438026.
25. Cai Z, Pan B, Jiang H, Zhang L. Chondrogenesis of human adipose-derived stem cells by in vivo co-graft with auricular chondrocytes from microtia. *Aesthetic Plast Surg.* 2015;39:431–439.
26. Zhang L, He A, Yin Z, et al. Regeneration of human-ear-shaped cartilage by co-culturing human microtia chondrocytes with BMSCs. *Biomaterials* 2014;35:4878–4887.
27. Remya NS, Nair PD. Engineering cartilage tissue interfaces using a natural glycosaminoglycan hydrogel matrix: An in vitro study. *Mater Sci Eng C Mater Biol Appl.* 2013;33:575–582.
28. Rosenzweig DH, Chicatun F, Nazhat SN, Quinn TM. Cartilaginous constructs using primary chondrocytes from continuous expansion culture seeded in dense collagen gels. *Acta Biomater.* 2013;9:9360–9369.
29. Scotti C, Mangiavini L, Boschetti F, et al. Effect of in vitro culture on a chondrocyte-fibrin glue hydrogel for cartilage repair. *Knee Surg Sports Traumatol Arthrosc.* 2010;18:1400–1406.
30. Flynn LE. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials* 2010;31:4715–4724.
31. Vondermark K, Gauss V, Vondermark H, Muller P. Relationship between cell-shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. *Nature* 1977;267:531–532.
32. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the

chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol.* 1997;17:2336–2346.
33. Yang Z, Huang CY, Candiotti KA, et al. Sox-9 facilitates differentiation of adipose tissue-derived stem cells into a chondrocyte-like phenotype in vitro. *J Orthop Res.* 2011;29:1291–1297.
34. Sbitany H, Serletti JM. Acellular dermis-assisted prosthetic breast reconstruction: A systematic and critical review of efficacy and associated morbidity. *Plast Reconstr Surg.* 2011;128:1162–1169.
35. Zelen CM, Orgill DP, Serena TE, et al. Human reticular acellular dermal matrix in the healing of chronic diabetic foot ulcerations that failed standard conservative treatment: A retrospective crossover study. *Wounds* 2017;29:39–45.
36. Meretoja VV, Dahlin RL, Wright S, Kasper FK, Mikos AG. Articular chondrocyte redifferentiation in 3D co-cultures with mesenchymal stem cells. *Tissue Eng Part C Methods* 2014;20:514–523.
37. Acharya C, Adesida A, Zajac P, et al. Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation. *J Cell Physiol.* 2012;227:88–97.
38. Bian L, Zhai DY, Mauck RL, Burdick JA. Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage. *Tissue Eng Part A* 2011;17:1137–1145.
39. Lee JS, Im GI. Influence of chondrocytes on the chondrogenic differentiation of adipose stem cells. *Tissue Eng Part A* 2010;16:3569–3577.
40. Pleumeekers MM, Nimeskern L, Koevoet JLM, Karperien M, Stok KS, van Osch GJVM. Trophic effects of adipose-tissue-derived and bone-marrow-derived mesenchymal stem cells enhance cartilage generation by chondrocytes in co-culture. *PLoS One* 2018;13:e0190744.
41. Hendriks JAA, Miclea RL, Schotel R, et al. Primary chondrocytes enhance cartilage tissue formation upon co-culture with a range of cell types. *Soft Matter* 2010;6:5080–5088.
42. Mo XT, Guo SC, Xie HQ, et al. Variations in the ratios of co-cultured mesenchymal stem cells and chondrocytes regulate the expression of cartilaginous and osseous phenotype in alginate constructs. *Bone* 2009;45:42–51.
43. Tsuchiya K, Chen GP, Ushida T, Matsuno T, Tateishi T. The effect of coculture of chondrocytes with mesenchymal stem cells on their cartilaginous phenotype in vitro. *Mat Sci Eng C-Bio S* 2004;24:391–396.
44. Moller T, Amoroso M, Hagg D, et al. In vivo chondrogenesis in 3D bioprinted human cell-laden hydrogel constructs. *Plast Reconstr Surg Glob Open* 2017;5:e1227.
45. Zhou G, Jiang H, Yin Z, et al. In vitro regeneration of patient-specific ear-shaped cartilage and its first clinical application for auricular reconstruction. *EBioMedicine* 2018;28:287–302.