Cartilage Regeneration in the Head and Neck Area: Combination of Ear or Nasal Chondrocytes and Mesenchymal Stem Cells Improves Cartilage Production

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Background: Cartilage tissue engineering can offer promising solutions for restoring cartilage defects in the head and neck area and has the potential to overcome limitations of current treatments. However, to generate a construct of reasonable size, large numbers of chondrocytes are required, which limits its current applicability. Therefore, the authors evaluate the suitability of a combination of cells for cartilage regeneration: bone marrow–derived mesenchymal stem cells and ear or nasal chondrocytes.

Methods: Human bone marrow–derived mesenchymal stem cells were encapsulated in alginate hydrogel as single-cell–type populations or in combination with bovine ear chondrocytes or nasal chondrocytes at an 80:20 ratio. Constructs were either cultured in vitro or implanted directly subcutaneously into mice. Cartilage formation was evaluated with biochemical and biomechanical analyses. The use of a xenogeneic coculture system enabled the analyses of the contribution of the individual cell types using species-specific gene-expression analyses.

Results: In vivo, human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes or human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes contained amounts of cartilage components similar to those of constructs containing chondrocytes only (i.e., bovine ear and nasal chondrocytes). In vitro, species-specific gene-expression analyses demonstrated that aggrecan was expressed by the chondrocytes only, which suggests a more trophic role for human bone marrow–derived mesenchymal stem cells. Furthermore, the additional effect of human bone marrow–derived mesenchymal stem cells was more pronounced in combination with bovine nasal chondrocytes.

Conclusions: By supplementing low numbers of bovine ear or nasal chondrocytes with human bone marrow–derived mesenchymal stem cells, the authors were able to engineer cartilage constructs with properties similar to those of constructs containing chondrocytes only. This makes the procedure more feasible for future applicability in the reconstruction of cartilage defects in the head and neck area because fewer chondrocytes are required. (*Plast. Reconstr. Surg.* 136: 762e, 2015.)

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Cartilage defects in the head and neck area are commonly encountered problems in reconstructive surgery. Currently, these defects are reconstructed with autologous cartilage grafts or artificial implants. Although autologous cartilage

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grafting has been used successfully, the procedure requires a high degree of surgical expertise, is associated with limited availability of autologous cartilage, and can cause severe donor-site morbidity. In addition, the use of artificial implants as an alternative is questioned in the head and neck area, because implants in this area are prone to induce a foreign body reaction and frequently lead to extrusion.[1] Cartilage tissue engineering can offer a promising solution for restoring missing or damaged cartilage and has the potential to overcome limitations of current treatments, reestablishing unique biological and functional properties of the tissue.

To generate a construct of reasonable size, large numbers of cells are required. Currently, cartilage tissue engineering is predominantly based on the use of two distinct cell types: chondrocytes and mesenchymal stem cells. However, each cell type is associated with specific disadvantages. Chondrocytes of several anatomical locations have been investigated for their applicability.[2–19] However, to obtain sufficient numbers of autologous cells, culture expansion seems an inevitable step in chondrocyte-based cartilage repair, resulting in generally more fibrous and mechanically inferior cartilage.[20] Mesenchymal stem cells, in contrast, are easily available from several tissues, can undergo multiple population doublings without losing their chondrogenic potential, and have the capacity to differentiate into cartilage tissue under appropriate culture conditions.[21–25] However, their use is currently debated, as the formed cartilage tissue is unstable and predisposed to tissue mineralization and ossification in vivo.[26–29] Considered together, the individual use of chondrocytes or mesenchymal stem cells is at present not ideal for cell-based cartilage repair in the head and neck area.

At present, the combination of both cell sources holds great promise for cartilage tissue engineering, as it reduces the required number of chondrocytes and extenuates most disadvantages of both individual cell types. In addition, mixed-cell cultures of chondrocytes and mesenchymal stem cells have been demonstrated to improve chondrogenesis[30] and to reduce hypertrophy and tissue mineralization.[31,32] Moreover, by decreasing the amount of chondrocytes (≤20 percent of the total cell mixture), culture expansion is no longer necessary, which allows the use of freshly isolated primary chondrocytes, leading to improved cartilage formation.[33] Moreover, by using primary cells, the procedure is more translatable toward a one-step clinical application.

To date, most research on mixed-cell–based cartilage repair has been performed with chondrocytes obtained from articular cartilage. So far, little research has been executed on mixed-cell cultures of mesenchymal stem cells and nonarticular chondrocytes, such as ear[34–36] or nasal chondrocytes.[37] Nonetheless, the translation of such basic research into a one-step clinical application is still unfeasible, primarily because these studies made use of nonoptimal culture conditions, such as the use of culture-expanded chondrocytes[34,35,37] or the paradoxical use of additional growth factors.[34,37] Moreover, only a few studies have yet evaluated the cartilage-forming capacity of mesenchymal stem cells/ear chondrocytes[34,36] and mesenchymal stem cells/nasal chondrocytes (none) in vivo. In an attempt to translate experimental research toward a one-stage cell-based cartilage repair procedure for cartilage defects in the head and neck area, the capacity, both in vitro and in vivo, of bone marrow–derived mesenchymal stem cells mixed with primary ear or nasal chondrocytes was studied. The formation of functional and stable nonmineralized cartilage was evaluated, along with the relative contribution of each individual cell population (i.e., chondrocytes, mesenchymal stem cells) to mixed-cell–based cartilage repair.

MATERIALS AND METHODS

Chemicals were obtained from Sigma-Aldrich (St. Louis, Mo.) unless stated otherwise.

Cell Sources

To obtain primary bovine chondrocytes from ear and the cartilaginous part of the nasoseptal origin, macroscopically intact cartilage was harvested from calves aged 6 months or younger, and washed with saline after careful resection of the perichondrium (i.e., three pools of three donors). To isolate cells, cartilage pieces were incubated for 1 hour with 2 mg/ml protease, followed by overnight incubation with 1.5 mg/ml collagenase B (Roche Diagnostics, Germany) in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, N.Y.). Nonexpanded primary chondrocytes were harvested and directly cultured in three-dimensional alginate hydrogel.

Human bone marrow–derived mesenchymal stem cells were isolated from bone marrow heparinized aspirates, after informed consent had been acquired and with approval of the local medical ethics committee (MEC-2004–142 and Albert Schweitzer Hospital 2011/7) ($n = 3$: male, 67 years; female, 75 years; and male, 22 years)

and seeded and cultured overnight in medium consisting of Minimum Essential Medium Alpha (Gibco), supplemented with fetal calf serum, L-ascorbic acid 2-phosphate, and 1 ng/ml basic fibroblast growth factor 2 (AbD Serotec, Kidlington, United Kingdom). Second-passage cells were harvested and cultured in three-dimensional alginate hydrogel.

Chondrogenesis

For three-dimensional alginate culture, cells were suspended at a density of 4×10^6 cells/ml in clinical grade 1.1% low-viscosity alginate solution dissolved in 0.9% sodium chloride (Batch MG-004; CellMed, Alzenau, Germany) as single-cell–type populations or as a combination of 80 percent human bone marrow–derived mesenchymal stem cells and 20 percent bovine ear or nasal chondrocytes (Table 1).

Flat constructs (8 mm diameter; 2 mm height) were processed as described previously.[19] Constructs were either cultured in vitro or implanted directly subcutaneously into mice. In vitro culture was performed for either 3 or 5 weeks in growth factor–free medium consisting of Dulbecco's Modified Eagle Medium supplemented with sodium pyruvate (Gibco), L-proline, supplemented insulin transferrin selenium (BD Biosciences, San Jose, Calif.), dexamethasone, and L-ascorbic acid 2-phosphate. Medium was changed two times per week. After 3 and 5 weeks, constructs were processed for biochemical and gene-expression analysis.

For in vivo studies, a total of 16 female 9-week-old NMRI nu/nu mice (Charles River Laboratories, Wilmington, Mass.) were used. Two separate incisions were made along the central line of the spine, after which four separate subcutaneous dorsal pockets were prepared by blunt dissection. After 8 weeks, animals were killed and samples were explanted for histologic, biomechanical, and biochemical analyses. Animal experiments were carried out with approval of the animal ethical committee (Erasmus MC 2429).

Biochemical Evaluation of the Extracellular Matrix

Alginate constructs were digested overnight at 56°C in papain (250 μg/ml in 0.2 M sodium hydrogen carbonate, 0.01 M ethylenediaminetetraacetic acid, containing 5 mM L-cysteine; pH 6.0). After digestion, samples were subjected to biochemical analyses to determine the DNA, glycosaminoglycan, and hydroxyproline content, as described previously.[19] In short, the amount of DNA was determined by ethidium bromide (Gibco), using calf thymus DNA as a standard. Sulfated glycosaminoglycans were quantified by the 1,9-dimethylmethylene blue dye-binding assay (pH 1.75), using shark chondroitin sulphate C as a standard. For the hydroxyproline content, digests were hydrolyzed, dried, and redissolved in 150 μl of water. The hydroxyproline content was measured using chloramine-T and dimethylaminobenzaldehyde as reagents and hydroxyproline (Merck, Darmstadt, Germany) as a standard.

Histologic Evaluation of the Extracellular Matrix

After 8 weeks of subcutaneous implantation, constructs were harvested, set in 2% agarose, fixed in 4% formalin in phosphate-buffered saline, and embedded in paraffin. Paraffin-embedded sections (6 μm) were deparaffinized and rehydrated.

To allow the use of monoclonal mouse antibody collagen type II (II-II6B3 1:100; Developmental Studies Hybridoma Bank, Iowa City, Iowa) on constructs that had been implanted in mice, the primary antibody was precoupled overnight with goat anti-mouse biotin at 4°C (1:500; The Jackson Laboratory, Bar Harbor, Me.), followed by a 2-hour incubation in 0.1% normal mouse serum (CLB, Amsterdam, The Netherlands) to prevent

Table 1. Construct Conditions

	Human Stem Cells		Bovine Chondrocytes	
Condition	Source	Cell Density ($\times 10^6$ cells/ml)*	Source	Cell Density ($\times 10^6$ cells/ml)*
hBMSC	hBMSCs	4	—	—
bEC	—	—	bECs	4
bNC	—	—	bNCs	4
hBMSC/bEC	hBMSCs	3.2	bECs	0.8
hBMSC/bNC	hBMSCs	3.2	bNCs	0.8
Control bEC	—	—	bECs	0.8
Control bNC	—	—	bNCs	0.8

hBMSCs, human bone marrow–derived mesenchymal stem cells; bECs, bovine ear chondrocytes; bNCs, bovine nasal chondrocytes.
*Cell density is displayed as the number of cells in 1 ml of alginate.

unwanted binding of the anti-mouse antibodies to mouse immunoglobulins.[38]

Antigen retrieval was performed through incubation with 0.1% pronase for 30 minutes at 37°C, continued with a 30 minutes' incubation with 1% hyaluronidase at 37°C. Nonspecific binding sites were blocked with 10% goat serum and sections were stained with the pretreated antibodies for 60 minutes. Sections were then incubated with enzyme-streptavidin conjugate (Label, 1:100, HK-321-UK; BioGenex Laboratories, San Ramon, Calif.) in phosphate-buffered saline/1% bovine serum albumin, followed by incubation with Neu Fuchsin substrate (Chroma, Kongen, Germany).

Biomechanical Analysis

For mechanical characterization, constructs 2.0 mm thick and 5 mm in diameter were created. The samples were placed in close-fitting 5-mm-diameter stainless steel cylindrical wells. Mechanical testing was performed with a materials testing machine (Zwick Z005; Zwick, Ulm, Germany) equipped with a 10-N load cell; a built-in displacement control; and a cylindrical, plane-ended, stainless steel indenter (diameter, 1.2 mm). Stress-strain testing was performed: the samples were compressed to a final height of 0.5 mm at a loading rate of 5 mm/minute. An in-house Matlab (MathWorks, Natick, Mass.) script was used to locate the sample surface and measure the sample thickness. Force-displacement curves were then converted to stress-strain curves. Measurements of compressive modulus at 40 percent strain (E40 percent) were determined.

Gene-Expression Analyses

To further evaluate the contribution of each individual cell type (i.e., human bone marrow–derived mesenchymal stem cells, and bovine ear or nasal chondrocytes) to cartilage matrix formation, species-specific gene-expression analysis was performed. For total RNA isolation, alginate was dissolved in ice-cold, 55 mM sodium citrate and 20 mM ethylenediaminetetraacetic acid in 150 mM sodium chloride and centrifuged. Each cell pellet was subsequently suspended in 1 ml of RNA-BeeTM (Tel-Test, Inc., Frindswood, Texas). RNA was extracted with chloroform and purified from the supernatant using the RNAeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines by on-column DNA digestion. Total RNA of each sample was reverse transcribed into cDNA using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Sankt Leon-Rot, Germany).

For quantitative real-time polymerase chain reaction analysis, forward and reverse primers were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, Calif.) to meet TaqMan or SYBR Green requirements. Analyzed genes are listed in Table 2. Real-time polymerase chain reaction was performed using TaqMan Universal PCR Mastermix (Applied Biosystems) or qPCR Mastermix Plus for SYBR Green (Eurogentec, Liège, Belgium) according to manufacturers' guidelines and using ABIPRISM 7000 with SDS software version 1.7 (Applied Biosystems). Relative gene expressions were calculated by means of the $2^{-}\Delta\Delta^{Ct}$ formula.

Statistical Analysis

All data were analyzed with PSAW statistics 20.0 (IBM Corp., Armonk, N.Y.). The mean and standard deviation were presented. In vitro data represent at least three independent donors per condition performed in triplicate. For statistical evaluation of these experiments, a mixed linear model was used, followed by Fisher's least significant post hoc comparisons tests. "Condition" and "time point" were defined as fixed factors in the model. "Donor" and "sample number" were treated as random factors. For the in vivo experiments, six constructs per condition were used, with duplicate samples for three independent donors. For statistical evaluation of these experiments, one-way analysis of variance was used followed by Fisher's least significant difference post hoc comparisons tests. For all tests, values of $p < 0.05$ were considered statistically significant.

Table 2. Sequences of Primers and Probes for Quantitative Real-Time Polymerase Chain Reaction

Genes	Primers and Probes
Human-specific genes	
hsGAPDH	
Forward	AGCTCACTGGCATGGCCTTC
Reverse	CGCCTGCTTCACCACCTTCT
hsACAN	
Forward	CAGCCACCACCTACAAACGCAG
Reverse	CTGGGTGGGATGCACGTCAGC
Bovine-specific genes	
bsGAPDH	
Forward	GTCAACGGATTTGGTCGTATTGGG
Reverse	TGCCATGGGTGGAATCATATTGG
bsACAN	
Forward	GGACACTCCTTGCAATTTGAGAA
Reverse	CAGGGCATTGATCTCGTATCG

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *ACAN*, aggrecan; *hs*, human-specific; *bs*, bovine-specific.

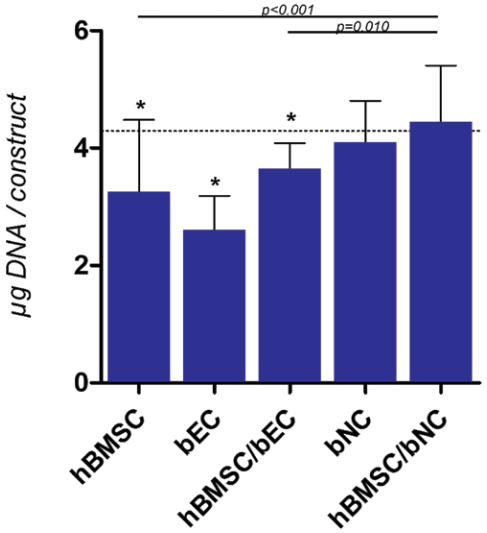

Fig. 1. Cell content of constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 3 weeks after in vitro culture. DNA content was determined at baseline before culture (*dotted line*), being on average 4.29 ± 0.96 µg DNA per construct, and after 3 weeks of culture. *$p < 0.05$ compared to baseline. Data are shown as mean ± SD. For statistical evaluation, a mixed model was used followed by a Fisher's least significant post hoc comparisons test. *hBMSC*, human bone marrow–derived mesenchymal stem cells ($n = 3$ experiments with 3 different donors); *bEC*, bovine ear chondrocytes ($n = 3$ experiments with 3 pools of donors); *bNC*, bovine nasal chondrocytes ($n = 3$ experiments with 3 pools of donors). For each experiment, three samples were used for analyses.

RESULTS

Cartilage Formation In Vitro

After 3 weeks, DNA content significantly decreased over time in constructs containing human bone marrow–derived mesenchymal stem cells ($p = 0.019$), bovine ear chondrocytes ($p = 0.010$), or human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes ($p < 0.001$), but remained stable in bovine nasal chondrocytes or human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes (Fig. 1). Similar results were obtained after 5 weeks (data not shown).

Because constructs were cultured in the absence of chondrogenic factors, constructs containing solely human bone marrow–derived mesenchymal stem cells produced very little glycosaminoglycan (Fig. 2, *above*) and collagen (Fig. 2, *below*). To demonstrate the additional effect of human bone marrow–derived mesenchymal stem cells in mixed-cell–type populations, a control condition—containing similar numbers of chondrocytes without the supplementation of human bone marrow–derived mesenchymal stem cells—was evaluated (Fig. 2, *white lines*). The additional effect of human bone marrow–derived mesenchymal stem cells in mixed-cell–type populations was dependent on the chondrocyte source used: the addition of human bone marrow–derived mesenchymal stem cells to bovine nasal chondrocytes demonstrated a significant increase in the production of glycosaminoglycan ($p = 0.012$) and collagen ($p = 0.007$) compared to their controls; no additional effects were observed in human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes. Human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes contained significantly more glycosaminoglycan ($p = 0.026$) and collagen ($p = 0.040$) compared with human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes. Normalization of the data to their initial number of seeded primary chondrocytes revealed more distinct differences between mixed-cell–type and single-cell–type populations: human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes and human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes produced more cartilage matrix per initial seeded chondrocyte than chondrocytes only (Fig. 2, *right*). Similar results were obtained after 5 weeks. [**See Figure, Supplemental Digital Content 1,** which shows cartilage matrix formation in constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 5 weeks after in vitro culture. Biochemical evaluation of the glycosaminoglycan (*GAG*) (*above*) and collagen (*below*) content, 5 weeks after culture in alginate. (*Left*) Amount of matrix components per construct. (*Right*) Graphs normalized to the initial number of seeded primary chondrocytes. A control condition—containing similar amounts of chondrocytes without supplementation of human bone marrow–derived mesenchymal stem cells—was evaluated to determine the additional effect of human bone marrow–derived mesenchymal stem cells on chondrocytes in cocultures (*white line*). *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ compared with the control condition. Data are shown as mean ± SD. For statistical evaluation, a mixed model was used followed by a Fisher's least significant post hoc comparisons test. *PC*, primary chondrocytes; *hBMSC*, human bone marrow–derived mesenchymal stem cells ($n = 3$ donors); *bEC*, bovine ear chondrocytes ($n = 3$ pooled donors); *bNC*, bovine nasal chondrocytes ($n = 3$ pooled donors). For each donor, three samples were used for analyses,

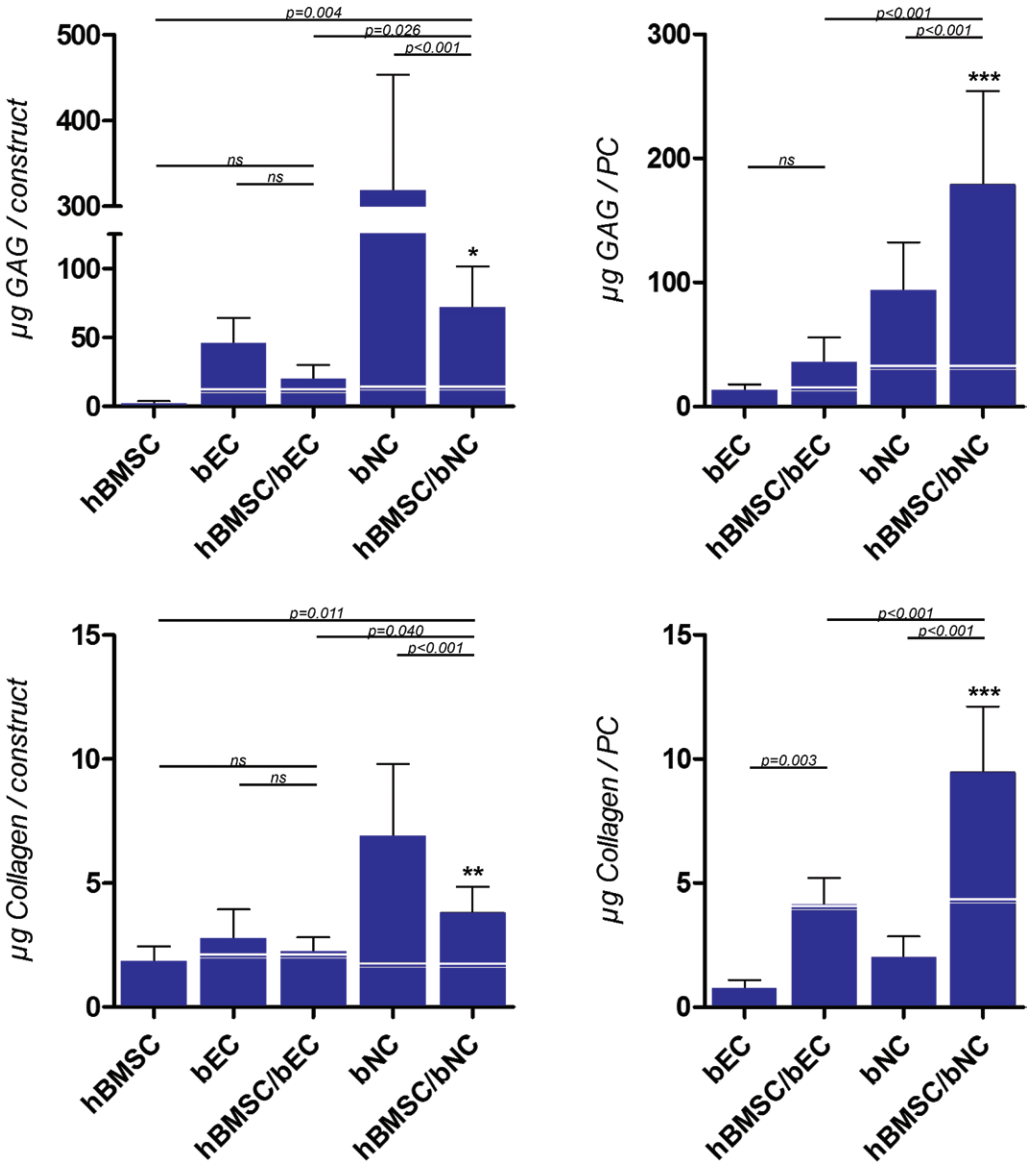

Fig. 2. Cartilage matrix formation in constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 3 weeks after in vitro culture. Biochemical evaluation of the glycosaminoglycan (*GAG*) (*above*) and collagen (*below*) content, 3 weeks after culture in alginate. (*Left*) Amount of matrix components per construct. (*Right*) Graphs showing matrix production normalized to the initial number of seeded primary chondrocytes. A control condition—containing similar amounts of chondrocytes (0.8×10^6 cells/ml) without supplementation of human bone marrow–derived mesenchymal stem cells—was evaluated to determine the additional effect of human bone marrow–derived mesenchymal stem cells (3.2×10^6 cells/ml) on chondrocytes in cocultures (*white line*). $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ compared with the control condition. Data are shown as mean ± SD. For statistical evaluation, a mixed model was used followed by a Fisher's least significant post hoc comparisons test. *PC*, primary chondrocytes; *hBMSC*, human bone marrow–derived mesenchymal stem cells ($n = 3$ experiments with three different donors); *bEC*, bovine ear chondrocytes ($n = 3$ experiments with three different donors); *bNC*, bovine nasal chondrocytes ($n = 3$ experiments with three different donors). For each experiment, three samples were used for analyses.

http://links.lww.com/PRS/B493.] These results demonstrate that human bone marrow–derived mesenchymal stem cells have an additional effect on chondrocytes in mixed-cell–type populations in vitro, in particular in combination with bovine nasal chondrocytes.

Cell Behavior in Cocultures

Using a xenogeneic culture system enabled us to determine the contribution of each individual cell type (i.e., human bone marrow–derived mesenchymal stem cells, and bovine ear or nasal chondrocytes) to cartilage matrix production using species-specific gene-expression analyses. First, *GAPDH* gene expression was analyzed after 5 weeks of in vitro culture. Human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes and human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes contained cells from both bovine (ear or nasal chondrocytes) and human (bone marrow–derived mesenchymal stem cells) origin (Table 3). Then, chondrogenic gene expression was analyzed by the *ACAN* gene. In a growth factor–free environment, human bone marrow–derived mesenchymal stem cells hardly expressed *hsACAN*. Besides, *hsACAN* was hardly expressed in human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes or human bone marrow–derived mesenchymal stem cells/ bovine nasal chondrocytes either. Conversely, human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes or human bone marrow–derived mesenchymal stem cells/ bovine nasal chondrocytes—containing solely 20 percent bovine chondrocytes—expressed as much or even higher levels of *bsACAN* compared to their 100 percent controls: human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes versus bovine ear or nasal chondrocytes, 3.96 ± 6.13-fold change; and human bone marrow–derived mesenchymal stem cells/ bovine nasal chondrocytes versus bovine ear or nasal chondrocytes, 4.56 ± 6.18-fold change. These data indicate that the formed cartilage matrix was from chondrocyte origin, which suggests a more trophic role for human bone marrow–derived mesenchymal stem cells herein.

Cartilage Formation In Vivo

After 8 weeks of implantation, all constructs were identified and harvested. Constructs containing bovine nasal chondrocytes or human bone marrow–derived mesenchymal stem cells/ bovine nasal chondrocytes resembled cartilage tissue in both color and texture, whereas constructs containing human bone marrow–derived mesenchymal stem cells, bovine ear chondrocytes, or human bone marrow–derived mesenchymal stem cells/ bovine ear chondrocytes were still fragile and did not express a cartilaginous appearance (Fig. 3, *above*). After subcutaneous implantation, none of the constructs had mineralized or ossified. Collagen type II was abundantly present in constructs containing bovine ear or nasal chondrocytes (both single-cell– and mixed-cell–type populations), but was not visible in constructs containing human bone marrow–derived mesenchymal stem cells only (Fig. 3, *below*).

Table 3. Gene-Expression Analyses 5 Weeks after In Vitro Culture*

Condition	Housekeeping Genes	
	hsGAPDH	*bsGAPDH*
hBMSC	24.1 ± 2.78	ND
bEC	ND	25.0 ± 2.4
bNC	ND	23.4 ± 1.7
hBMSC/bEC	24.0 ±1.7	26.0 ± 2.7
hBMSC/bNC	24.8 ± 1.7	25.1 ± 2.4

ND, not detected (Ct value > 35.00); *hsGAPDH*, human-specific *GAPDH*; *bsGAPDH*, bovine-specific *GAPDH*; *h*BMSC, human bone marrow–derived mesenchymal stem cells (n = 3 experiments with three different donors); *b*EC, bovine ear chondrocyte (n = 3 experiments with three different donors); *b*NC, bovine nasal chondrocyte (n = 3 experiments with three different donors).
*Data are shown as mean Ct values ± SD of housekeeping genes. For each experiment, three samples were used for analyses.

In vivo, human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes and human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes contained similar quantities of cartilage matrix as constructs containing chondrocytes only. Moreover, human bone marrow–derived mesenchymal stem cells/ bovine nasal chondrocytes produced significantly more glycosaminoglycan (p = 0.004) compared with human bone marrow–derived mesenchymal stem cells/ bovine ear chondrocytes (Fig. 4, *above*). Collagen production demonstrated a similar trend, albeit without statistically significant differences (Fig. 4, *below*). Normalization of the data to their initial number of seeded primary chondrocytes revealed more distinct differences between mixed-cell–type and single-cell–type populations: human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes produced significantly more glycosaminoglycan and collagen per initial seeded primary chondrocyte compared with bovine nasal chondrocytes and human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes ($p < 0.05$) (Fig. 4, *right*).

After subcutaneous implantation, the elastic modulus was highest in constructs containing bovine nasal chondrocytes, although large variation between samples was observed (Fig. 5).

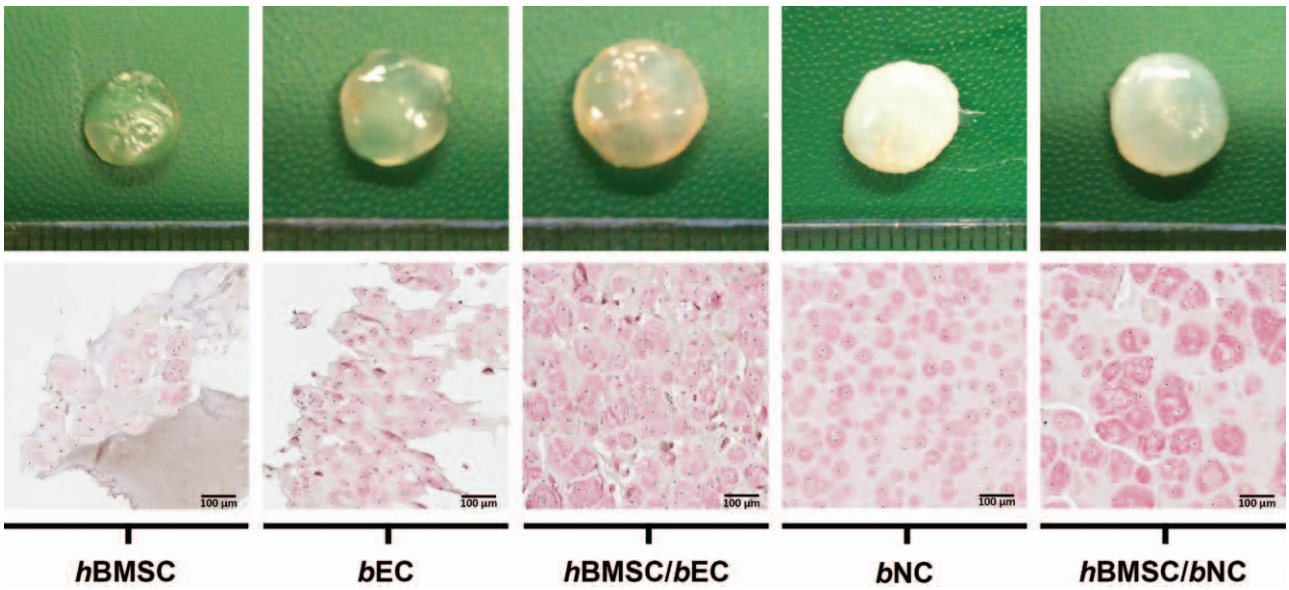

Fig. 3. Cartilage matrix formation in constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 8 weeks after subcutaneous implantation into mice. Macroscopic appearance (*above*) of cartilage constructs and a collagen type II immunohistochemical staining (*below*) 8 weeks after subcutaneous implantation. *hBMSC*, human bone marrow–derived mesenchymal stem cells ($n = 2$ experiments with two different donors); *bEC*, bovine ear chondrocyte ($n = 2$ experiments with three pools of donors); *bNC*, bovine nasal chondrocyte ($n = 2$ experiments with three pools of donors). For each experiment, two samples were used for analyses.

DISCUSSION

The combination of chondrocytes and mesenchymal stem cells holds great promise for cell-based cartilage repair in the head and neck area, as it reduces the required number of chondrocytes and extenuates most disadvantages of individually used cell types such as culture-expanded chondrocytes or mesenchymal stem cells. Mixed-cell cultures have been demonstrated to improve chondrogenesis[30] and to reduce hypertrophy and tissue mineralization.[31,32] Unfortunately, most research on mixed-cell–based cartilage repair was performed with articular chondrocytes. So far, little research in this field has been performed on nonarticular chondrocytes, such as ear chondrocytes[34–36] or nasal chondrocytes.[37] This study evaluates the two most relevant cell sources for cell-based cartilage repair in the head and neck area—ear and nasal chondrocytes—and replaced 80 percent of the chondrocytes with human bone marrow–derived mesenchymal stem cells. In line with previous studies on mixed-cell–based cartilage repair, human bone marrow–derived mesenchymal stem cells/bovine ear chondrocytes or human bone marrow–derived mesenchymal stem cells/bovine nasal chondrocytes produced similar quantities of cartilage matrix components as constructs containing chondrocytes only. Moreover, the cartilage tissue formed seemed stable and did not calcify in vivo. This suggests that 80 percent of the chondrocytes can be replaced by human bone marrow–derived mesenchymal stem cells without influencing cartilage matrix production and stability. Therefore, mixed cultures of bone marrow–derived mesenchymal stem cells and ear or nasal chondrocytes could be very advantageous for cell-based cartilage repair in the head and neck area, as appropriate numbers of cells are more easily acquired from bone marrow aspirates than from cartilage biopsy specimens.

By using primary cells, we aimed to translate the procedure toward a single-stage clinical application. Currently, for articular cartilage repair, two clinical trials are already designed as single-stage procedures.[39,40] Unfortunately, the little research performed on mixed-cell cultures using bone marrow–derived mesenchymal stem cells and ear chondrocytes[34–36] or nasal chondrocytes[37] impeded the translation of such basic research to clinical application, because these studies made use of nonoptimal culture conditions. First, instead of using primary chondrocytes, most research[34,35,37] was performed with culture-expanded chondrocytes, which requires a two-stage procedure: (1) a surgical procedure to harvest cartilage tissue for chondrocyte isolation and further culture expansion; and (2) a surgical procedure to implant the cell-based cartilage graft. Second, others

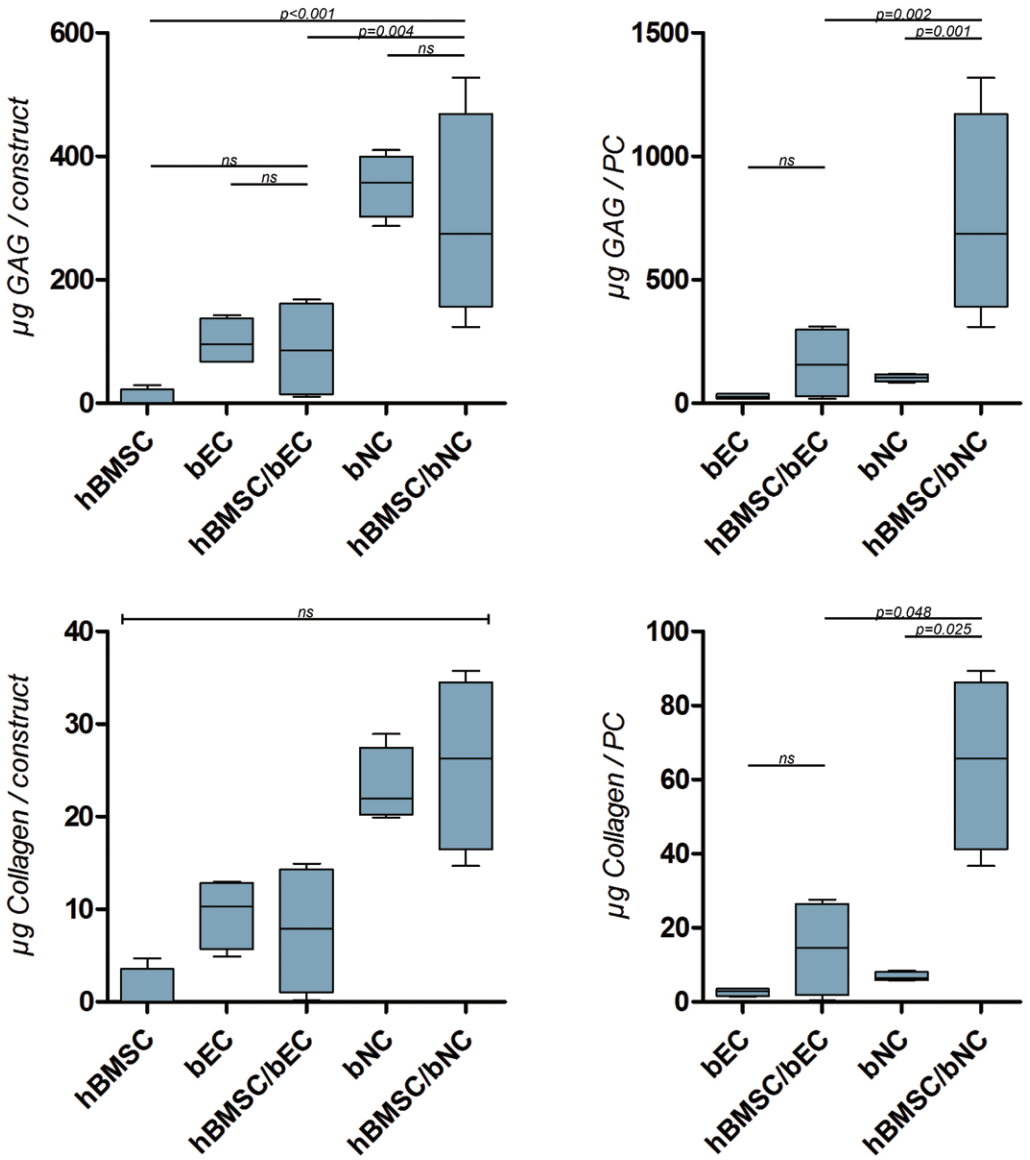

Fig. 4. Cartilage matrix formation in constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 8 weeks after subcutaneous implantation into mice. Biochemical evaluation of the glycosaminoglycan (*GAG*) (*above*) and collagen (*below*) content, 8 weeks after subcutaneous implantation. (*Left*) Amount of matrix components per construct. (*Right*) Graphs showing matrix production normalized to the initial number of seeded primary chondrocytes. Data are shown as box-and-whisker plots. For statistical evaluation, a one-way analysis of variance was used followed by a Fisher's least significant difference post hoc comparisons test. *PC*, primary chondrocytes; *hBMSC*, human bone marrow–derived mesenchymal stem cells (n = 2 experiments with two different donors); *bEC*, bovine ear chondrocytes (n = 2 experiments with three pools of donors); *bNC*, bovine nasal chondrocytes (n = 2 experiments with three pools of donors). For each experiment, two samples were used for analyses.

have cultured their constructs in growth factor–enriched medium.[34,37] Because growth factors stimulate the redifferentiation and differentiation of both culture-expanded chondrocytes and bone marrow–derived mesenchymal stem cells, the use of growth factors might have interfered with the underlying mechanisms of cell-to-cell interaction in their culture system. Moreover, clinical use of growth factors is limited by the problem of adequate delivery[41] and the requirement of special regulatory approval by the U.S. Food and Drug Administration or European Medicines Agency. Finally, so far, only a few studies have evaluated the cartilage-forming capacity of

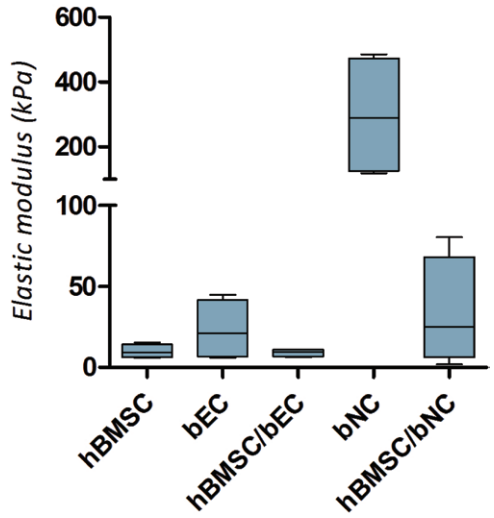

Fig. 5. Biomechanical behavior in constructs containing human bone marrow–derived mesenchymal stem cells and/or chondrocytes, 8 weeks after subcutaneous implantation into mice. Biomechanical properties were determined 8 weeks after subcutaneous implantation. Data are shown as box-and-whisker plots. *PC*, primary chondrocytes; *hBMSC*, human bone marrow–derived mesenchymal stem cells (n = 2 experiments with two different donors); *bEC*, bovine ear chondrocytes (n = 2 experiments with three pools of donors); *bNC*, bovine nasal chondrocytes (n = 2 experiments with three pools of donors). For each experiment, two samples were used for analyses.

bone marrow–derived mesenchymal stem cells/ear chondrocytes[34,36] and bone marrow–derived mesenchymal stem cells/nasal chondrocytes (none) in vivo. Therefore, in an attempt to translate experimental research toward a single-stage clinical application in the future, we have studied the in vitro and in vivo capacity of human bone marrow–derived mesenchymal stem cells mixed with primary bovine ear or nasal chondrocytes cultured in a growth factor–free environment.

We made use of a xenogeneic culture system (i.e., bovine chondrocytes, human bone marrow–derived mesenchymal stem cells). The species mismatch did not impede cartilage formation, confirming previously published results of human bone marrow–derived mesenchymal stem cells combined with xenogeneic chondrocytes.[30,42–44] Moreover, by making use of a xenogeneic culture system, we were able to determine the contribution of each individual cell type to cartilage matrix production using species-specific gene-expression analyses. In this way, we proved that cartilage matrix formation originated from bovine chondrocytes and that human bone marrow–derived mesenchymal stem cells fulfilled a trophic role herein. Although numerous cellular communication pathways have been hypothesized to explain the beneficial effect of mixed cultures,[32] this outcome was in accordance with previous studies, investigating the effect of mesenchymal stem cells on articular chondrocytes.[30,44–46] We found no evidence that paracrine soluble factors released by chondrocytes enhanced the chondrogenic differentiation of human bone marrow–derived mesenchymal stem cells, as stated by others.[47–51] Although the importance of juxtacrine or gap-junctional signaling is still unclear in the literature,[46] our mixed cells encapsulated in alginate hydrogels implicated that such signaling pathways are of less importance than paracrine signaling pathways, because the alginate hydrogel hinders direct cell-to-cell contact.

Besides the trophic effect of human bone marrow–derived mesenchymal stem cells on chondrocytes, we demonstrated that this effect was also dependent on the chondrocyte source used. The differences between the chondrocyte sources was most obvious in the in vitro experiments: bovine nasal chondrocytes were clearly stimulated by human bone marrow–derived mesenchymal stem cells, whereas bovine ear chondrocytes were not at all influenced by them. Although the in vivo experiments showed a positive effect of human bone marrow–derived mesenchymal stem cells on both bovine ear and nasal chondrocytes, it was obvious that the use of bovine nasal chondrocytes led to constructs with a higher amount of glycosaminoglycan and collagen and higher equilibrium modulus than bovine ear chondrocytes. Clear subtype-specific differences in cartilage-forming potential is in accordance with our previously published work, confirming that ear and nasal chondrocytes have unique gene-expression profiles inducing dissimilar proliferation capacity, cartilage matrix formation, and elastin fiber deposition.[5,19]

Before this method can be successfully applied as a one-step clinical application, there are some limitations to overcome. First, the elastic modulus after 8 weeks of subcutaneous implantation was low and approximately 1 percent of that of native human ear or nasal cartilage.[52] Although the biomechanical properties of the constructs were rather low, alginate enabled a homogeneous cell distribution and prevented cells from floating out while permitting nutrient diffusion and oxygen transfer to the cells to create an environment to form new cartilage matrix with sufficient properties.[53] Therefore, injected into a mechanically stable scaffold, alginate could be an excellent cell-carrying gel for future cell-based cartilage

repair. Second, the cell density used in this study might not be optimal for obtaining engineered tissue that is clinically applicable. Our experimental setup did not allow us to further increase cell density because of limitations in the number of cells available. Nevertheless, it allowed us to study the interactions between the cell types. For clinical application, it would be ideal to use only low numbers of human primary chondrocytes supplemented with human bone marrow–derived mesenchymal stem cells. We have combined human bone marrow–derived mesenchymal stem cells and chondrocytes at a 4:1 ratio, as the effect of human bone marrow–derived mesenchymal stem cells on articular chondrocytes was already studied by us at such a ratio. Although others have used a 4:1 ratio for their research as well,[30,54] no consensus on optimal ratios of bone marrow–derived mesenchymal stem cells to chondrocytes has been established for ear and nasal chondrocytes. Future research needs to clarify whether we could further reduce the amount of primary chondrocytes without inhibiting cartilage matrix production. Finally, for future clinical application, the use of allogeneic human bone marrow–derived mesenchymal stem cells can be considered, as mesenchymal stem cells have been demonstrated to be immune privileged.[55] Alternatively, instead of using culture-expanded human bone marrow–derived mesenchymal stem cells, use of the mononuclear fraction of freshly isolated autologous bone marrow might even be considered.[56]

CONCLUSIONS

In summary, this study demonstrates that constructs containing a combination of 80 percent human bone marrow–derived mesenchymal stem cells and 20 percent bovine ear or nasal chondrocytes produced similar quantities of cartilage matrix components as constructs containing chondrocytes only. Therefore, 80 percent of the chondrocytes can easily be replaced by human bone marrow–derived mesenchymal stem cells without influencing cartilage matrix production. Using this procedure, the chondrocytes need no culture expansion in vitro, supporting the use of a one-stage cell-based cartilage repair procedure for cartilage defects in the head and neck area.

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