ARTHRITIS & RHEUMATISM
Vol. 58, No. 1, January 2008, pp 197–208
DOI 10.1002/art.23155

Engineered Cartilage Generated by Nasal Chondrocytes Is Responsive to Physical Forces Resembling Joint Loading

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Objective. **To determine whether engineered cartilage generated by nasal chondrocytes (ECN) is responsive to different regimens of loading associated with joint kinematics and previously shown to be stimulatory of engineered cartilage generated by articular chondrocytes (ECA).**

Methods. **Human nasal and articular chondrocytes, harvested from 5 individuals, were expanded and cultured for 2 weeks into porous polymeric scaffolds. The resulting ECN and ECA were then maintained under static conditions or exposed to the following loading regimens: regimen 1, single application of cyclic deformation for 30 minutes; regimen 2, intermittent application of cyclic deformation for a total of 10 days, followed by static culture for 2 weeks; regimen 3, application of surface motion for a total of 10 days.**

Results. **Prior to loading, ECN constructs contained significantly higher amounts of glycosaminoglycan (GAG) and type II collagen compared with ECA constructs. ECN responded to regimen 1 by increasing collagen and proteoglycan synthesis, to regimen 2 by increasing the accumulation of GAG and type II collagen as well as the dynamic modulus, and to regimen 3 by increasing the expression of superficial zone protein, at the messenger RNA level and the protein level, as well as the release of hyaluronan. ECA constructs were overall less responsive to all loading regimens, likely due to the lower extracellular matrix content.**

Conclusion. **Human ECN is responsive to physical forces resembling joint loading and can up-regulate molecules typically involved in joint lubrication. These findings should prompt future in vivo studies exploring the possibility of using nasal chondrocytes as a cell source for articular cartilage repair.**

Cell-based therapies currently in clinical application for the treatment of articular cartilage lesions typically rely on the use of autologous chondrocytes harvested from a small biopsy specimen of articular cartilage. A cartilage biopsy specimen from a joint, even if harvested from a non–load-bearing site, represents an additional injury to the cartilage surface and has been reported to be detrimental to the surrounding healthy articular cartilage (1). To overcome this problem, several groups of investigators proposed the use of mesenchymal progenitor cells from bone marrow (2–4), synovial membrane (5–8), or periosteum (9,10) or the use of chondrocytes from nonarticular cartilage, such as the ear (11,12), rib (11), or nasal septum (11,13).

Nasal cartilage would be a particularly interesting source of cells, because the tissue is characterized as a hyaline cartilage and contains differentiated chondrocytes expressing the collagen types typical of articular cartilage (14). Biopsy specimens of nasal cartilage can be harvested under local anesthesia and by a procedure that is less invasive than removing tissue from specific areas of the joint. Morbidity associated with nasal cartilage biopsy is also reduced, because the donor site is not subjected to high levels of physical force (15). Indeed, nasal septal cartilage (sometimes in substantial

Supported in part by the European Union (Project FP6 STEPS, contract NMP3-CT-2005-500465), the Swiss National Science Foundation (grant 3200B0-110054), and the "Deutsche Arthrose-Hilfe e.V."

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Submitted for publication May 21, 2007; accepted in revised form September 21, 2007.

amounts) is routinely resected during septoplasty operations, with few sequelae or complications (15).

Several studies have indicated that human nasal septum chondrocytes, even after culture expansion and associated dedifferentiation, retain the capacity to redifferentiate and generate hyaline-like tissue (11,13,16), with mechanical properties approaching those of native cartilage (17,18). In a comparative study using human articular chondrocytes from age-matched donors, it was also established that nasal chondrocytes proliferate faster and have a higher and more reproducible chondrogenic capacity, both in vitro and in an ectopic model in vivo (13). Furthermore, although chondrogenesis by expanded human articular chondrocytes has the tendency to decrease with donor age (19), the quality of engineered cartilage generated by human nasal chondrocytes (ECN) does not appear to be dependent on the age of the donor (16).

In order for nasal chondrocytes to be considered for implantation at an articular cartilage site, it is crucial to determine not only their chondrogenic capacity but also whether they are responsive to forces that are typically associated with joint loading. However, to the best of our knowledge, nothing is yet known about whether nasal chondrocytes and articular chondrocytes, which have a different embryologic origin and are physiologically exposed to a markedly different biochemical and biomechanical environment, share a similar mode of response to physical forces.

The goal of this study was to assess the response of human nasal chondrocytes to different regimens of loading, using experimental setups and readout parameters previously shown by different groups of investigators to enhance matrix synthesis or production of lubrication proteins by articular chondrocytes, with statically cultured specimens as controls. In particular, we applied the following modalities of loading: 1) a single application of cyclic deformation for 30 minutes (20), 2) intermittent application of cyclic deformation for a total of 10 days (21), or 3) intermittent application of surface motion for a total of 10 days (22). As a model to investigate cell response to loading, we engineered cartilage tissue obtained by seeding and culturing expanded chondrocytes into 3-dimensional (3-D) porous scaffolds. The response of ECN was compared with that of engineered cartilage generated by articular chondrocytes (ECA), using cells isolated from different sites in the same individuals.

MATERIALS AND METHODS

Chondrocyte isolation, expansion, and culture in 3-D scaffolds. Specimens of healthy articular and nasal cartilage tissue were harvested postmortem from, respectively, full-thickness biopsy specimens of the femoral condyle and the nasal septum of 5 individuals (mean age 42 years [range 32–60 years]), in accordance with the local ethics committee. Articular chondrocytes and nasal chondrocytes were isolated by 22 hours of incubation at 37°C in 0.15% type II collagenase and resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4.5 mg/ml D-glucose, 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, 100 m*M* HEPES buffer, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamate (complete medium).

Nasal chondrocytes and articular chondrocytes were plated in culture dishes at a density of 10^4 cells/cm^2 and expanded in complete medium supplemented with 1 ng/ml of transforming growth factor β1 (TGFβ1), 5 ng/ml of fibroblast growth factor 2, and 10 ng/ml of platelet-derived growth factor BB. This specific combination of growth factors was previously shown to enhance the proliferation of nasal chondrocytes (11) and articular chondrocytes (19), as well as their postexpansion chondrogenic differentiation. When subconfluent, cells were detached by sequential treatment with 0.3% type II collagenase and 0.05% trypsin/0.53 m*M* EDTA, replated at 5×10^3 cells/cm^2, and cultured until subconfluency was reached again, corresponding to a total of ~8–10 population doublings.

Expanded chondrocytes were statically seeded into nonwoven meshes (3.7×10^6 cells in 6-mm–diameter, 2-mm–thick disks) made of esterified hyaluronan (HA) (HYAFF11; Fidia Advanced Biopolymers, Abano Terme, Italy) or into foams (10×10^6 cells in 8-mm–diameter, 4-mm–thick disks) made of porous polyurethane (22,23). Cell–scaffold constructs were cultured for 2 weeks under static conditions in complete medium supplemented with 0.1 m*M* ascorbic acid, 10 μg/ml insulin, and 10 ng/ml TGFβ3 (differentiating medium). The engineered cartilage tissues generated by nasal or articular chondrocytes (ECN and ECA, respectively) were either assessed histologically, biochemically and mechanically, as detailed below, or further cultured using 1 of the 3 regimens of physical loading described below, with static controls maintained under free-swelling conditions.

Application of physical force to engineered cartilage tissue. *Regimen 1: single application of cyclic deformation.* ECN and ECA constructs based on HYAFF11 meshes were exposed to a single application of sinusoidal deformation for 30 minutes (15% amplitude strain superimposed on a 15% strain offset, at 0.1 Hz frequency). The regimen was adapted from that used in a previous study (20) and was applied using a previously described bioreactor system (24). Briefly, constructs were placed in the peripheral wells of culture chambers, and cylindrical plungers were precisely positioned in contact with the top surfaces of each construct by using micrometer screws, until an increase in the measured load was observed. One separate chamber, allocating 6 engineered constructs, was used for each of 4 experimental groups (i.e., ECN or ECA exposed to loading or maintained in a control free-swelling condition). Cells from 4 different donors were used in 4 independent

experiments. After loading, the constructs were assessed for proteoglycan and collagen synthesis, as described below.

Regimen 2: intermittent application of cyclic deformation. ECN and ECA constructs based on HYAFF11 meshes were exposed to intermittent application of sinusoidal deformation for 30 minutes (7.5% amplitude strain superimposed on 7.5% strain offset, at 0.1 Hz frequency), every second day for 10 days. The amplitude and frequency were derived from previous studies and were reported to be stimulatory of proteoglycan synthesis in articular cartilage explants (25,26) and also in engineered cartilage, if it was sufficiently developed (21). The duration of the application was modified according to the protocol used by Waldman et al (20).

Constructs (6 per experimental group) were placed in the bioreactor chambers described above, under gentle mixing (30 revolutions per minute), as previously described (24). After each 30-minute application, the plungers were raised 500 μm over the constructs, in order to allow for recovery of the full thickness and to reduce mass transport limitations. The construct–plunger contact position was reestablished before each application of loading. Unloaded constructs were cultured in the same chambers in free-swelling conditions as control. Differentiating medium was changed twice a week and collected for determination of the amount of glycosaminoglycan (GAG) released. Constructs were assessed histologically, biochemically, and mechanically, as described below, either immediately after the last loading cycle or following an additional 2 weeks of static culture in differentiating medium. Cells from 5 different donors were used in 5 independent experiments.

Regimen 3: intermittent application of surface motion. ECN and ECA constructs based on polyurethane foams were attached to specimen holders using bone cement (Norian Skeletal Repair System; Norian, Cupertino, CA) and exposed to intermittent surface motion by oscillation of a ceramic hip ball over their surface for 30 minutes ($\pm 60°$ amplitude rotation superimposed on 10% strain offset, at 0.1 Hz frequency), every second day for 10 days, using a previously described bioreactor system (22). Between the loading periods, constructs did not have any contact with the ball. Unloaded constructs were cultured in free-swelling conditions as a control.

The response of engineered cartilage to surface motion was assessed for what was previously reported to be the predominant effect on bovine articular chondrocytes cultured on polyurethane scaffolds, namely, the expression of genes encoding for lubricating proteins (22). Differentiating medium was changed daily and collected for quantification of the amount of superficial zone protein (SZP) and HA released. Immediately after the last cycle of loading, constructs were assessed for the messenger RNA (mRNA) expression of SZP, HA synthase 1 (HAS-1), and HAS-2, as described below. Cells from 4 different donors were used in 4 independent experiments.

Analytic techniques. *Proteoglycan and collagen synthesis.* Immediately after the single application of cyclic loading (regimen 1), constructs were incubated for 24 hours in the presence of L-^{3}H-proline (5 μCi/construct) and ^{35}S-SO_4 (4 μCi/construct) to quantify the amount of newly synthesized collagen and proteoglycans, respectively. After radioactive labeling, the amount of newly synthesized collagen and proteoglycans was quantified both in medium (released fraction), as previously described (20), and in the constructs (accumulated fraction), following digestion with proteinase K (0.5 ml of 1 mg/ml proteinase K in 50 m*M* Tris with 1 m*M* EDTA, 1 m*M* iodoacetamide, and 10 μg/ml pepstatin A) for 15 hours at 56°C. The amount of newly synthesized components was normalized to the DNA content of the constructs (determined as described below).

Biochemical analyses. For determination of GAG and DNA, constructs were digested with proteinase K as described above. The amount of GAG was measured spectrophotometrically after reaction with dimethylmethylene blue (27), with chondroitin sulfate as a standard. DNA was measured spectrofluorometrically using the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard (28). The amount of GAG released from the constructs into the medium during the 10-day culture period was quantified from the media collected from each group and normalized to the mean DNA content of a single construct. For determination of type II collagen, constructs were digested with tosylamide-2-phenylethyl chloromethyl ketone–treated bovine pancreatic trypsin at 2 mg/ml (in 50 m*M* Tris HCl, pH 7.6), using an initial incubation of 15 hours at 37°C followed by a further 2 hours of incubation at 65°C after the addition of fresh trypsin. Samples were boiled for 15 minutes to inactivate the enzyme (29), and the amounts of type II collagen were assayed by inhibition enzyme-linked immunosorbent assay (ELISA) using a mouse IgG monoclonal antibody to denatured type II collagen (30).

Histologic and immunohistochemical analyses. Constructs were fixed in 4% formalin for 24 hours at 4°C, embedded in paraffin, and cross-sectioned (7 μm thick). Sections were stained with Safranin O for GAG or for type II collagen (antibody II-II6B3; Hybridoma Bank, University of Iowa, Iowa City), as previously described (31).

Mechanical analyses. Mechanical assessments of ECN and ECA from regimen 2 as well as of cell-free HYAFF11 scaffolds were performed in a standard miniature test instrument in unconfined compression (Synergie 100; MTS Systems, Eden Prairie, MN), within 4 hours after termination of the culture. The thickness of each specimen was measured first, by moving the crosshead down at a rate of 0.1 mm/second until a load was detected, and then the specimen was assessed to measure the following parameters.

Equilibrium modulus. Constructs were exposed to 5 incremental strains of 5% each, accomplished at a crosshead speed of 0.17 mm/second, and were considered fully relaxed after each strain increment when the rate of change in the load was lower than 0.001 N/second (corresponding to ~35 Pa/second). The equilibrium modulus was determined as the slope of the equilibrium load normalized to the specimen area, plotted versus the applied strain (32).

Pulsatile dynamic modulus. Constructs were subjected to 5 cycles of compressive loading/unloading at a rate of 0.17 mm/second, reaching a maximum of 20% strain and separated by a no-load period of time equal to that for loading, as previously described (33). The mean pulsatile dynamic modulus was computed by performing linear regressions through the

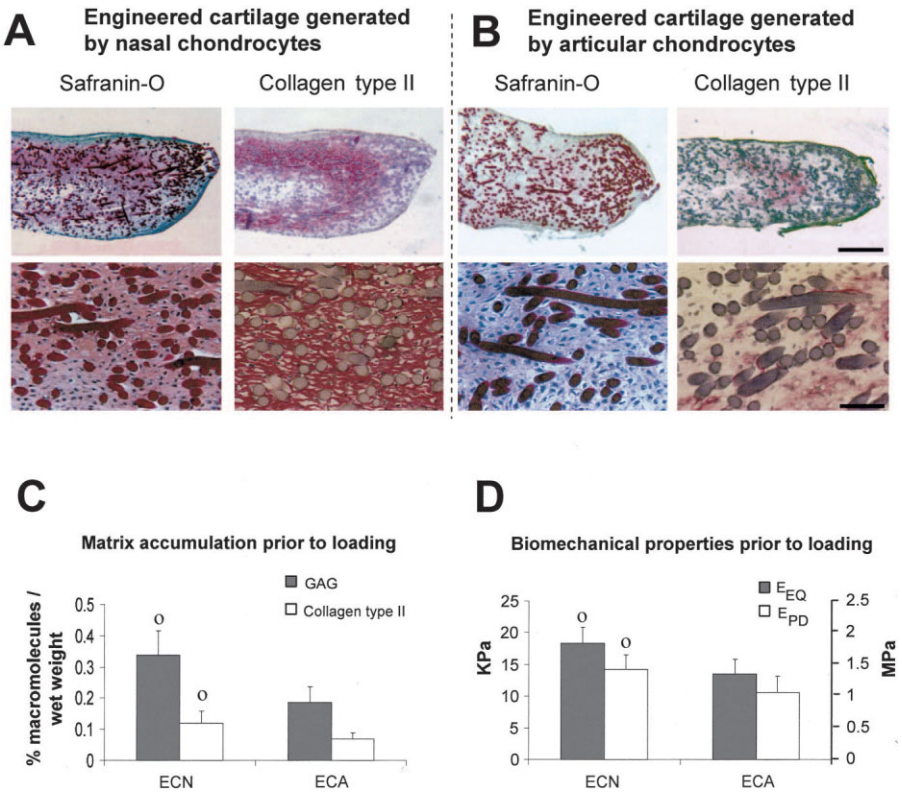

Figure 1. Properties of engineered cartilage tissue generated by nasal chondrocytes (ECN) or articular chondrocytes (ECA) prior to mechanical loading. **A** and **B,** Safranin O and type II collagen immunohistochemical staining of representative ECN or ECA after 2 weeks of culture in chondrogenic medium. Top rows (bar = 500 μm) show lower-magnification views of bottom rows (bar = 100 μm). **C,** Glycosaminoglycan (GAG) and type II collagen content of ECN and ECA. **D,** Equilibrium modulus (E_{EQ}, left y-axis) and pulsatile dynamic modulus (E_{PD}, right y-axis) of ECN and ECA. Values are the mean and SEM results from 5 independent experiments. o = significant difference versus ECA.

stress–strain curve of each cycle, outside the toe region. Because cartilage displays poroviscoelastic material properties, the cyclic nature of the tests means that the pulsatile dynamic modulus is actually a form of the compressive dynamic elastic modulus, which is the vector sum of the storage modulus (the in-phase, elastic component) and the loss modulus (the out-of-phase, poroviscous component).

Gene expression at the mRNA level. Gene expression analysis of ECN and ECA from regimen 3 was performed by real-time reverse transcription (RT)–polymerase chain reaction (PCR) (GeneAmp 5700; Applied Biosystems, Foster City, CA), following RNA extraction using TRI Reagent (Molecular Research Center, Cincinnati, OH) and RT with TaqMan reagents (Applied Biosystems, Foster City, CA). The oligonucleotide primers and fluorescent probes for human SZP, HAS-1, and HAS-2 were described previously (22). Primers and probes for amplification of 18S ribosomal RNA for use as endogenous control were from Applied Biosystems. Real-time RT-PCRs were not multiplexed.

Quantification of HA and SZP in culture media. The amounts of released HA and SZP were quantified in the media collected from loaded and free-swelling ECN and ECA constructs from regimen 3. The HA concentration was measured in a competitive ELISA system (Echelon Biosciences, Salt Lake City, UT). For analysis of SZP, media were fractionated by anion-exchange chromatography on a 1-ml column of Resource Q (Amersham Biosciences, Uppsala, Sweden) as described previously (34). SZP was then eluted, dialyzed, and separated by gel electrophoresis as previously described (22). Immunoreactive bands were detected with the ECL Plus Western blotting detection system (Amersham Biosciences), following incubation with the polyclonal antibody 06A10 (1:1,000 dilution; kindly provided by C. R. Flannery, Wyeth Research, Cambridge, MA) against human megakaryocyte-

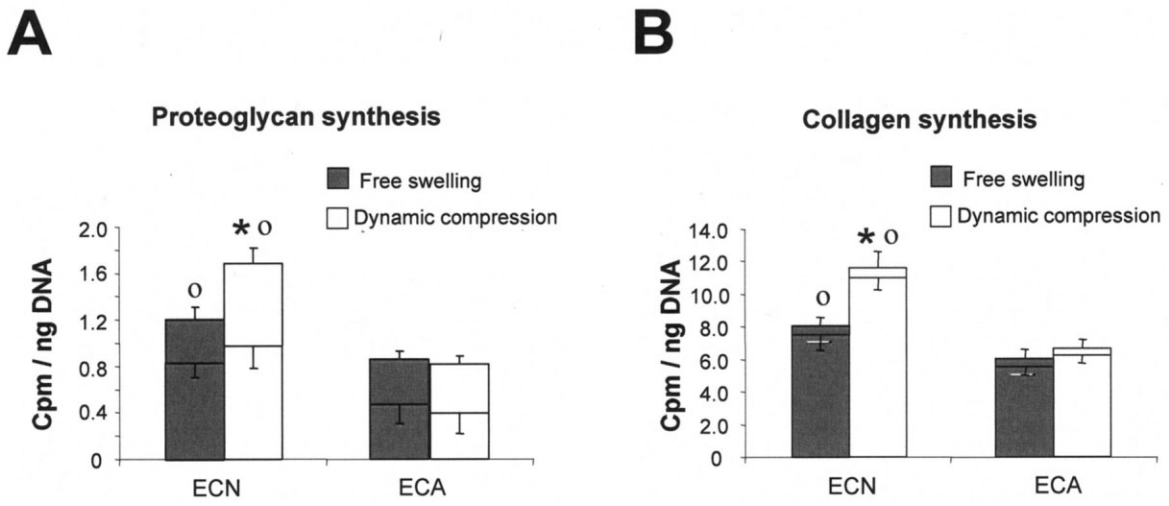

Figure 2. Biosynthetic activity of ECN or ECA following a single application of cyclic loading (regimen 1). Amounts of newly synthesized proteoglycan (**A**) and collagen (**B**) were measured by incorporation of $^{35}S\text{-}SO_4$ and ^{3}H-proline, respectively, in constructs maintained under free-swelling conditions or 24 hours following a single application of cyclic loading. The upper and lower parts of the columns represent newly synthesized molecules released in the medium or accumulated in the extracellular matrix, respectively. Values are the mean and SEM results from 4 independent experiments. o = significant difference versus ECA cultured under the same condition; * = significant difference versus the free-swelling condition of the same cell source. See Figure 1 for definitions.

stimulating factor, previously reported to be homologous to SZP or lubricin (34).

Statistical analysis. All values are presented as the mean ± SEM. Measurements were obtained from at least 4 independent experiments (i.e., with cells from at least 4 different donors). For each experiment and experimental group, at least duplicate specimens were assessed. Differences between experimental groups were statistically assessed by Wilcoxon's 2-tailed tests, using SigmaStat software version 13 (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Properties of the tissue-engineered cartilage prior to mechanical loading. After 2 weeks of culture using nonwoven HYAFF11 scaffolds, prior to mechanical loading, ECN constructs showed more intense and uniform staining for GAG and type II collagen than did ECA tissues (Figures 1A and B). Consistently, the biochemically quantified amounts of GAG and type II collagen were significantly higher (71% and 73%, respectively) for ECN compared with ECA tissues (Figure 1C). Mechanical tests indicated that the equilibrium modulus and the pulsatile dynamic modulus were also significantly higher (35% and 34%, respectively) for ECN than for ECA (Figure 1D).

Regimen 1: single application of cyclic deformation. The application of cyclic deformation for 30 minutes significantly increased synthesis of collagen and proteoglycans (44% and 39%, respectively) in ECN constructs, whereas no significant effect was measured in ECA constructs (Figure 2). In particular, in ECN tissue, the increased proteoglycan synthesis was reflected by a significantly higher release (86%) of the newly synthesized molecules, and the increased collagen synthesis resulted in a significantly higher accumulation (47%) of the newly synthesized molecules. As compared with ECA, ECN constructs had significantly higher proteoglycan and collagen synthesis after both free swelling and cyclic deformation. Interestingly, for both ECN and ECA, the percentage of newly synthesized proteoglycans released into the media (40–60%) was markedly higher than the corresponding percentage of newly synthesized collagen (5–6%).

Regimen 2: intermittent application of cyclic deformation. *Assessment after 10 days of cyclic deformation.* Intermittent application of cyclic deformation for 10 days induced a significant increase (66%) in the total

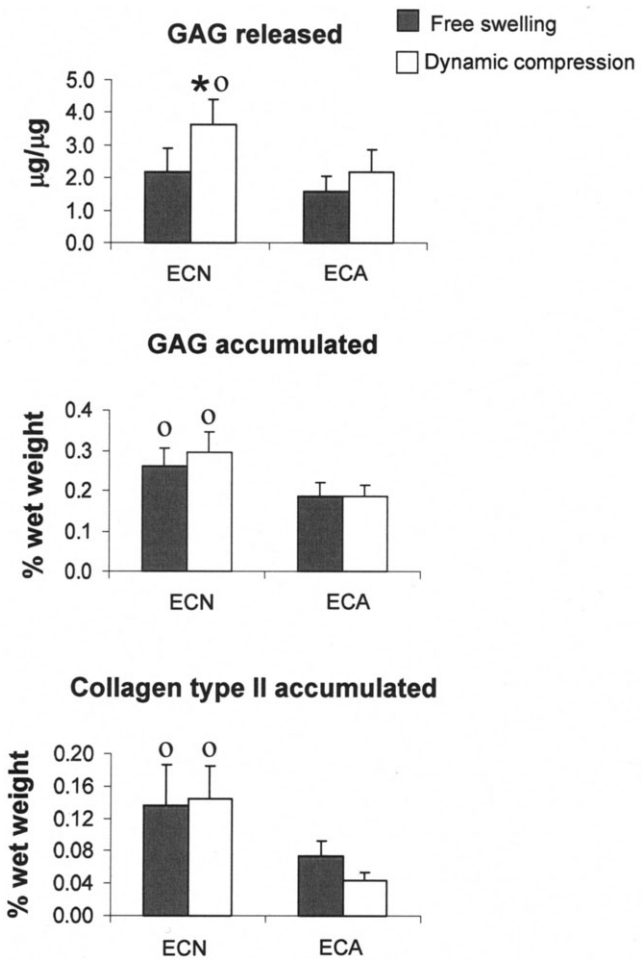

Figure 3. Properties of ECN and ECA after intermittent application of cyclic deformation for 10 days (regimen 2). Columns show the amounts of GAG released into the culture medium or the amount of GAG or type II collagen accumulated in the engineered cartilage, normalized to the DNA content or expressed as a percentage of tissue wet weight, respectively. Values are the mean and SEM results from 5 independent experiments. o = significant difference versus ECA; * = significant difference versus free swelling. See Figure 1 for definitions.

amount of GAG released by ECN constructs but not by ECA constructs (Figure 3), consistent with the results from regimen 1. The wet weight fractions of accumulated GAG and type II collagen were higher in ECN constructs than in ECA constructs and were not affected significantly by the application of loading in either group. These findings were consistent with the fact that loaded and free-swelling constructs displayed similar staining intensities for GAG and type II collagen, as well as similar mechanical properties (data not shown).

Assessment after 10 days of cyclic deformation followed by 2 weeks of static culture. In order to assess whether cyclic deformation would influence the development of engineered cartilage tissue after terminating its application, ECN and ECA constructs cultured under loading or free-swelling conditions for 10 days were statically cultivated for 2 additional weeks. At this stage, ECN constructs previously exposed to loading were stained at higher intensity and uniformity for both GAG and type II collagen as compared with the free-swelling controls (Figure 4A). Instead, no apparent effect of loading was observed for ECA constructs, where staining patterns were generally faint and scattered in discrete areas (Figure 4B). Biochemical quantification of GAG and type II collagen, as a percentage of the tissue wet weight, confirmed that 1) in ECN, cyclic deformation significantly increased the content of both molecules (by 55% and 60%, respectively), 2) in ECA, cyclic deformation had no significant effect on the content of either molecule, and 3) ECA constructs had significantly lower content of both molecules than did ECN tissue (Figure 4C). Mechanical properties were generally consistent with the tissue composition: ECN had significantly higher levels of equilibrium modulus and pulsatile dynamic modulus than ECA and, unlike that in ECA constructs, the response to loading in ECN constructs was an increase in the pulsatile dynamic modulus (by 33%, in 5 of 5 donors; $P = 0.04$) and the equilibrium modulus (by 26%, in 4 of 5 donors; $P = 0.08$) (Figure 4D).

We next investigated whether the increased response to cyclic deformation of ECN as compared with ECA could be related to the higher degree of tissue development at the time of loading. The fold difference in the GAG content of ECN and ECA constructs of different donors in response to loading was shown to be significantly positively correlated with the GAG content of the same constructs prior to loading (Figure 5). Thus, the constructs with the lowest GAG content had the most detrimental response to loading, whereas tissue with the highest GAG content had the most stimulatory response to loading. Importantly, ECN and ECA appeared to follow a similar trend.

Regimen 3: intermittent application of surface motion. *Effect of surface motion on mRNA expression of SZP, HAS-1, and HAS-2.* Intermittent application of surface motion to ECN constructs for 10 days induced a significant and marked increase (32.9-fold) in the mRNA expression of SZP but not HAS-1 and HAS-2. Only low levels and nonsignificant up-regulation of SZP

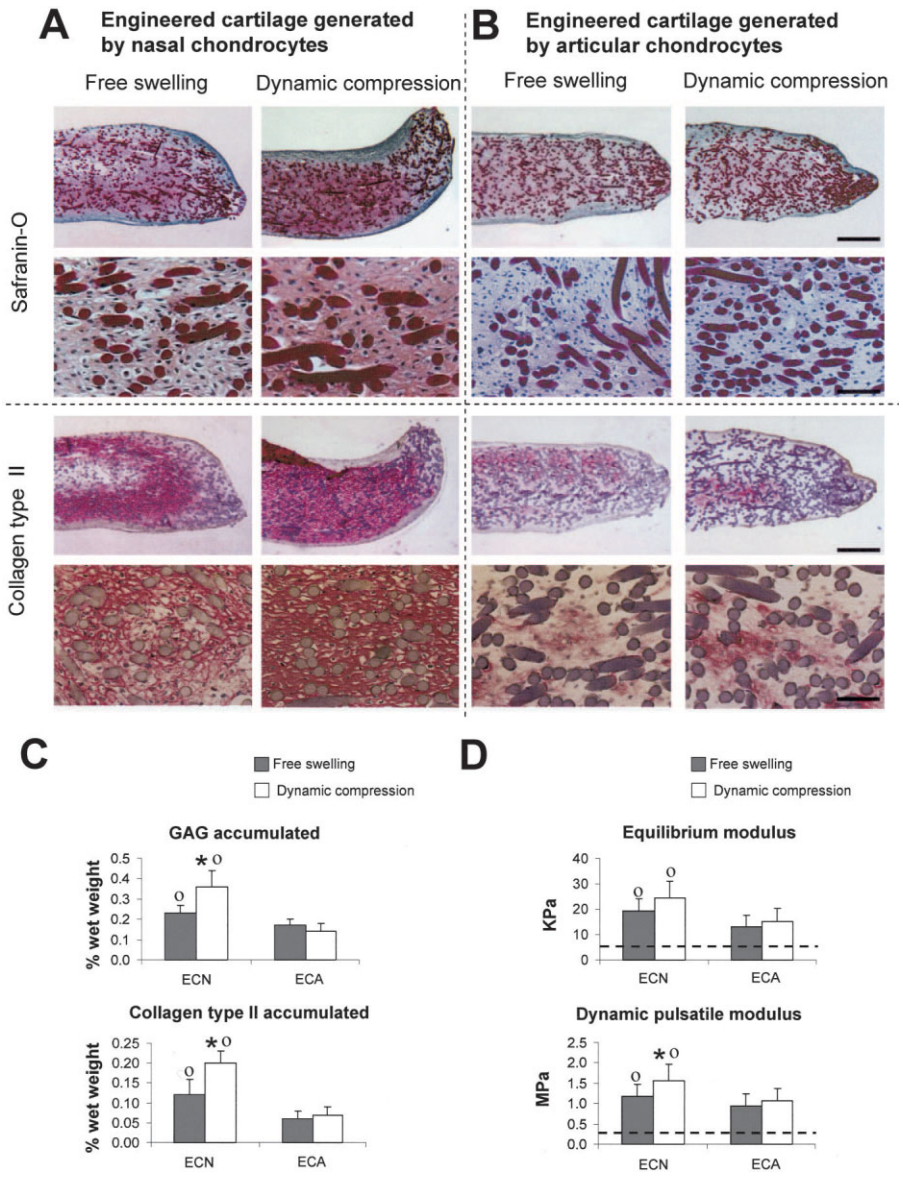

Figure 4. Properties of ECN or ECA after intermittent application of cyclic deformation for 10 days (regimen 2) followed by 2 weeks of static culture. **A** and **B,** Safranin O and type II collagen immunohistochemical staining of ECN or ECA constructs. Top rows (bar = 500 μm) show lower-magnification views of bottom rows (bar = 100 μm). **C,** Amounts of GAG and type II collagen accumulated, expressed as a percentage of tissue wet weight. **D,** Equilibrium modulus and pulsatile dynamic modulus of ECN and ECA constructs. Broken lines indicate the mechanical properties of the cell-free HYAFF11 scaffold. Values are the mean and SEM results from 5 independent experiments. o = significant difference versus ECA cultured under the same condition; $*$ = significant difference versus the free-swelling condition of the same cell source. See Figure 1 for definitions.

and HAS-1 mRNA (3.4-fold and 1.8-fold, respectively) were measured in ECA following application of surface motion. However, the expression of these genes remained higher in ECA constructs as compared with stimulated ECN constructs (15.4-fold for SZP and 51.9-fold for HAS-1) (Figure 6A). The expression of HAS-2

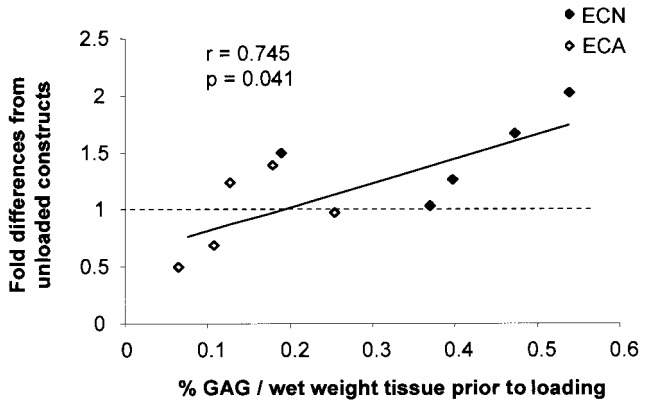

Figure 5. Response to loading of engineered cartilage versus the matrix content of the tissue. The response to loading (regimen 2 followed by 2 weeks of static culture) of the wet weight percentage of GAG, calculated as the fold difference from the unloaded constructs, was correlated with the GAG content at the time of loading. The *P* value was determined using Pearson's 2-tailed correlation test. See Figure 1 for definitions.

mRNA was similar in ECN and ECA tissue and was not regulated by surface motion.

Effect of surface motion on the release of SZP and HA in culture media. In the absence of loading, the secretion of SZP and HA protein into the culture medium by ECA cultures was significantly higher than that by ECN cultures (Figure 6B), although the difference was less marked than at the mRNA level. SZP and HA protein amounts released into the medium were enhanced by applied surface motion in both ECA and ECN constructs (for SZP, 1.8-fold and 1.4-fold, respectively; for HA, 1.3-fold and 1.4-fold, respectively). Consistent with the results for mRNA expression, even following application of surface motion, SZP and HA were released in larger amounts (1.8-fold and 2.1-fold, respectively) by ECA constructs than ECN constructs (Figure 6B).

DISCUSSION

In the present study, we demonstrate that human nasal chondrocytes, expanded and subsequently cultured into 3-D porous scaffolds, are responsive to physical forces resembling joint loading. In particular, cyclic deformation increased GAG and collagen synthesis and enhanced the mechanical properties of the resulting ECN. Moreover, surface motion up-regulated the expression of genes involved in joint lubrication, at both the mRNA level (i.e., for SZP) and the protein level (i.e., for SZP and HA).

The model used to investigate chondrocyte response to loading was based on the expansion (and thus dedifferentiation) of nasal chondrocytes and articular chondrocytes, followed by culture into HA- or polyurethane-based porous scaffolds for 2 weeks. Based on previously reported calculations, the extent of expansion would be sufficient to produce enough cells for clinically relevant graft sizes (i.e., a few square centimeters), starting from biopsy specimens weighing only a few milligrams (11). The culture time in scaffolds was previously reported to be sufficient to activate the process of cell redifferentiation (35) and to generate constructs with relevant mechanical properties (36), with the capacity to further develop in vivo (37). The resulting ECN was histologically, biochemically, and mechanically superior to ECA. This finding is consistent with a previous report that human nasal chondrocytes generally have a higher and more reproducible postexpansion chondrogenic capacity than articular chondrocytes (13), but this study is the first in which the analysis was performed by a paired comparison using cells harvested from different sites in the same individuals.

The different loading regimens used in the present study to characterize the response of human nasal chondrocytes to specific physical forces only remotely mimic the mechanical conditioning of joint loading and thus cannot be used to predict directly the capacity of nasal chondrocytes to respond to joint loading when implanted in an articular cartilage defect in patients. Conversely, the models used allowed us to investigate the intrinsic capacity of nasal chondrocytes of human origin to respond to defined and controlled physical conditioning, in a way that is decoupled by the complex interactions between biologic, biochemical, and biomechanical cues of a large-sized animal joint.

A single application of cyclic loading (regimen 1) induced a significant and immediate increase in collagen and proteoglycan synthesis by nasal chondrocytes. In contrast to a previous study in which a similar regimen was used (20), ECA constructs in this study were not responsive to a single application of cyclic deformation. The discrepancy could result from the fact that Waldman et al (20) used primary (i.e., nonexpanded) bovine articular chondrocytes from young animals, seeded directly as a thin layer on the top surface of the scaffolds, at a higher cell density. In our system, consisting of expanded human articular chondrocytes from adult individuals, seeded at a relatively lower density into the

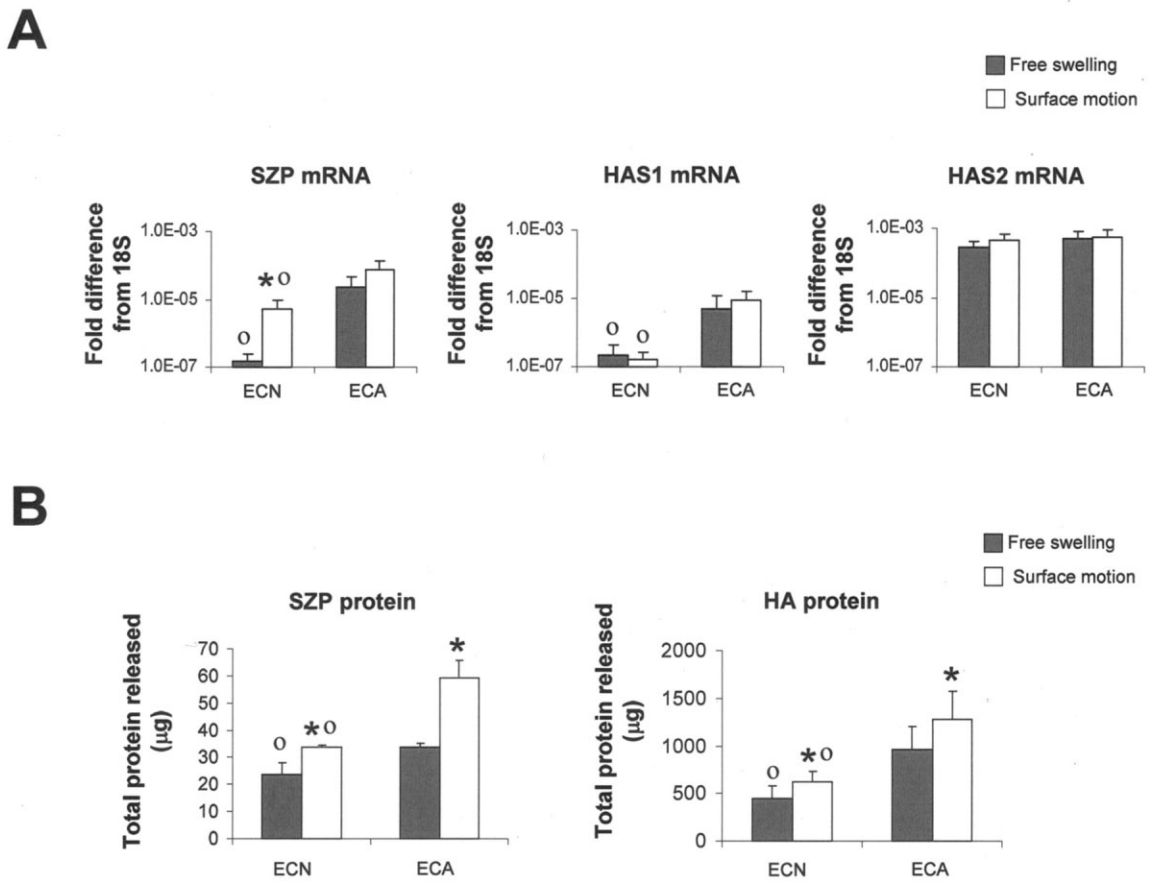

Figure 6. Messenger RNA expression and protein release of molecules involved in joint lubrication by ECN or ECA, following intermittent application of surface motion (regimen 3). **A,** Real-time reverse transcription–polymerase chain reaction analysis of the expression of superficial zone protein (SZP) and hyaluronan synthase 1 (HAS-1) and HAS-2 mRNA in ECN and ECA. E = exponential in base 10. **B,** Quantification of the total SZP and HA proteins released in the culture medium. Values are the mean and SEM results from 4 independent experiments. o = significant difference versus ECA cultured under the same condition; ∗ = significant difference versus the free-swelling condition of the same cell source. See Figure 1 for other definitions.

scaffolds, chondrogenesis is expected to proceed less efficiently, and thus the extracellular and pericellular matrices, which are critical to determine chondrocyte response to loading (21,38), were most likely less developed. This interpretation is consistent with the finding that the more developed constructs based on nasal chondrocytes were indeed responsive to the loading regimen.

Intermittent application of cyclic deformation for 10 days (regimen 2) increased the amount of GAG released from ECN during loading, possibly as a direct effect of increased convection (38,39) and/or as a consequence of increased activity of degradative enzymes (40). Because the amounts of accumulated GAG were not affected by loading, the higher amounts of GAG released indicate an overall increase in GAG synthesis by nasal chondrocytes, which is consistent with results obtained by application of regimen 1. A significant increase in GAG accumulation in ECN in response to loading was observed only following an additional 2 weeks of static culture. Considering that the net amount of accumulated GAG is critically determined by the combination of GAG synthesis and GAG release, the result may reflect that the effect of loading on GAG release decays more rapidly than that on GAG synthesis. A similar consideration could apply for type II collagen, although in this case the amounts released in medium were below levels of detection for the specific assay used

and thus could not be quantified. The increased accumulation of GAG and type II collagen is consistent with a significant increase in the pulsatile dynamic modulus (Figure 4D) and establishes that nasal chondrocytes are indeed positively responsive to a repeated application of cyclic loading.

The fact that cyclic loading of ECA did not significantly increase the amount of GAG released or accumulated, as well as the amount of type II collagen accumulated, may (as for regimen 1) be explained by the lower amount of matrix deposited at the time of loading. This would be consistent with previous findings that the response of engineered cartilage tissue to mechanical loading is also directly related to the stage of tissue development (21). In fact, we observed a significant and positive correlation in GAG deposition between the GAG content prior to loading and the response to loading (Figure 5), similar to what was previously described for ECA (21). Interestingly, the correlation was determined by combining data obtained using ECN and ECA, suggesting common matrix-mediated mechanisms of response to loading.

Intermittent application of surface motion for 10 days (regimen 3) to ECN induced a significant increase in the expression of SZP mRNA and the release of SZP and HA protein into the medium. This indicates that nasal chondrocytes exposed to a joint-specific modality of loading can up-regulate the expression of proteins considered to play a fundamental role in joint lubrication. The expression of HAS-1 and HAS-2, in both articular chondrocytes and nasal chondrocytes, was not regulated by the regimen of surface motion applied. The lack of response in those 2 genes by articular chondrocytes, in contrast to previous studies (22,41,42), could again result from differences in the cell source (i.e., human versus bovine), as well as by slight modifications of the loading protocol (i.e., lower frequency and number of cycles of stimulation).

The levels of SZP mRNA expression and protein release by nasal chondrocytes remained significantly lower than those by articular chondrocytes, confirming that the gene is associated with a cellular function typical of cells derived from the joint. Interestingly, however, expression levels significantly increased in ECN following application of surface motion and entered the range of those measured in ECA under free-swelling conditions. This finding indicates a certain plasticity of expanded nasal chondrocytes and the potential to adapt to a typical joint environment. Similar cell plasticity to joint kinematics regimens was recently observed in chondrocytes from the middle and deep zones of articular cartilage, which normally do not express SZP but were able to up-regulate its expression following stimulation by surface motion (43).

In this study, we did not address whether the capacity of nasal chondrocytes to respond to joint-specific mechanical forces was intrinsic in the isolated cells or was acquired following dedifferentiation. The latter possibility was advocated in a recent study, whereby rabbit expanded auricular chondrocytes (i.e., from elastic cartilage) implanted into the intervertebral discs of rabbits survived and produced extracellular matrix lacking elastic fibers and displaying hyaline-like characteristics (44). Despite the different ontogeny of nasal cartilage and articular cartilage (i.e., ectodermal versus endodermal) and their different topographic locations in the body, no markers specific of human cells resident in these 2 tissue types (i.e., expressed only by nasal chondrocytes or articular chondrocytes) are known. The identification of human cartilage subtype–specific genes will be instrumental to investigating whether nasal chondrocytes isolated from their physiologic tissue milieu and dedifferentiated in vitro would or would not maintain the biologic memory of their original environment.

In conclusion, the present study demonstrates that expanded human nasal chondrocytes cultured in a 3-D scaffold are responsive to different physical forces typical of a joint, and suggests that the resulting ECN, implanted in an articular cartilage defect, could further mature and improve the biochemical and mechanical properties under stimulation of the physical forces of the joint. Most obviously, further preclinical investigations are necessary to validate the possibility of using nasal chondrocytes as a cell source for articular cartilage repair.

ACKNOWLEDGMENTS

We are grateful to Anna Marsano, David Wendt, Francine Wolf (University Hospital Basel), and Robert Peter (AO Research Institute) for the technical support provided, and to Alma U. Daniels (University of Basel) for the helpful discussions on cartilage mechanics.

AUTHOR CONTRIBUTIONS

Dr. Martin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Candrian, Hollander, Jakob, Martin.

Acquisition of data. Candrian, Vonwil, Bonacina, Miot, Dickinson, Li.

Analysis and interpretation of data. Candrian, Barbero, Farhadi, Wirz, Martin.
Manuscript preparation. Candrian, Barbero, Alini, Heberer, Martin.
Statistical analysis. Candrian, Bonacina.

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