Editor's Summary

Cells from Nose Repair Tissue in Joint

Cartilage repair remains a yet unmet clinical need, with few viable cell therapy options available. Taking cells from the knee or ankle to repair worn cartilage requires additional surgery and, in turn, pain and healing for the patient. As such, a new, accessible cell source would greatly benefit these patients. Here, Pelttari and colleagues looked up the nose for cells that may have the capacity to regenerate cartilage. Nasal septum cells arise from the neuroectoderm—the tissue that gives rise to the nervous system—and are better at repairing tissues than their mesoderm counterparts. These regenerative capabilities have been attributed to a lack of homeobox (HOX) gene expression. The authors therefore investigated whether nasal chondrocytes (HOX-negative, neuroectoderm origin) were compatible with an articular cartilage environment, like the knee joint (HOX-positive, mesoderm origin). The authors discovered that adult human nasal chondrocytes were able to self-renew and also, to their surprise, adopt a HOX-positive profile upon implantation into a mesoderm environment; in goats, this led to repair of experimental articular cartilage defects. In an ongoing clinical trial, human nasal chondrocytes have been shown to be safe once transplanted, suggesting translation of this new, easy-to-access cell source for repairing damaged joints.

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Adult human neural crest–derived cells for articular cartilage repair

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In embryonic models and stem cell systems, mesenchymal cells derived from the neuroectoderm can be distinguished from mesoderm-derived cells by their Hox-negative profile—a phenotype associated with enhanced capacity of tissue regeneration. We investigated whether developmental origin and Hox negativity correlated with self-renewal and environmental plasticity also in differentiated cells from adults. Using hyaline cartilage as a model, we showed that adult human neuroectoderm-derived nasal chondrocytes (NCs) can be constitutively distinguished from mesoderm-derived articular chondrocytes (ACs) by lack of expression of specific Hox genes, including HOXC4 and HOXD8. In contrast to ACs, serially cloned NCs could be continuously reverted from differentiated to dedifferentiated states, conserving the ability to form cartilage tissue in vitro and in vivo. NCs could also be reprogrammed to stably express Hox genes typical of ACs upon implantation into goat articular cartilage defects, directly contributing to cartilage repair. Our findings identify previously unrecognized regenerative properties of Hox-negative differentiated neuroectoderm cells in adults, implying a role for NCs in the unmet clinical challenge of articular cartilage repair. An ongoing phase 1 clinical trial preliminarily indicated the safety and feasibility of autologous NC-based engineered tissues for the treatment of traumatic articular cartilage lesions.

INTRODUCTION

Although the first clinical report of cell-based cartilage repair by autologous chondrocyte transplantation now dates back more than 20 years (1), the predictable and durable regeneration of articular cartilage remains an unmet clinical need. Currently available cell-based techniques to treat hyaline articular cartilage mainly consist in the recruitment of mesenchymal stem/stromal cells (MSCs) from the subchondral bone (microfracturing) or in the grafting of ex vivo–expanded autologous articular chondrocytes (ACs). These techniques typically result in an unpredictable long-term outcome (2), likely related to the phenotypic instability of the cartilage tissue formed by MSCs (3) or the large inter-donor variability in the cartilage-forming capacity of ACs (4). To bypass the aforementioned critical issues, a more reproducibly chondrogenic cell source should be identified.

As compared to ACs, nasal chondrocytes (NCs) were shown to have a higher capacity to generate functional cartilaginous tissues, with lower donor-related dependency (5–7). NCs could respond similarly to ACs to physical forces resembling joint loading (8) and could efficiently recover after exposure to inflammatory factors typical of an injured joint (9). Moreover, NCs are easily accessible from a small biopsy of the nasal septum, with minimal donor site morbidity. NCs and ACs derive from tissues sharing a common hyaline nature and produce a similar pattern of extracellular matrix molecules. However, NCs and ACs originate from different germ layers, and the developmental or genetic compatibility of NCs with an articular cartilage environment has never been addressed.

Hox genes play a key role during development by encoding for transcription factors that control the three-dimensional (3D) body plan organization according to the rules of spatiotemporal colinearity (10, 11). Transplantation experiments in developing quail-chick embryos have demonstrated that the ability of implanted cells to be reprogrammed by environmental conditions is progressively restricted with the activation of Hox genes (12–15). In particular, it was demonstrated that Hox-positive neural crest–derived cells from posterior rhombomeres could not substitute for Hox-negative cells after transplantation into anterior domains, but by contrast, Hox-negative neural crest–derived cells could replace Hox-positive cells, leading to normal tissue formation (12). The terms “Hox-positive” and “Hox-negative” are here used for cells respectively expressing or not expressing defined sets of Hox genes. This principle was recently extended to an adult murine model, where it was shown that Hox-negative neuroectoderm-derived skeletal stem cells, but not Hox-positive mesoderm-derived skeletal stem cells, can adopt the Hox expression status of heterotopic transplantation sites, thereby leading to robust tissue repair (16). A Hox-negative status was also proposed to reflect a higher level of self-renewal capacity in totipotent human embryonic stem cells (17) and functionally distinct human stem cell populations derived from cord blood (18).

Here, using hyaline cartilage as a model, we first investigated whether Hox genes are differentially expressed in human NCs (neuroectodermal, and more specifically neural crest, origin) and ACs (mesoderm origin). We then assessed whether developmental origin and Hox negativity remain associated with self-renewal capacity and environmental plasticity in differentiated cells from adult tissues. Finally, we tested the compatibility of NC-based engineered tissues for articular cartilage repair by implantation in experimental defects in goats and by acquiring early observations in a pilot clinical trial. We report a previously unexpected capacity of Hox-negative, human adult NCs to self-renew in...
serial cloning assays and to be reprogrammed upon implantation in mesoderm environments by activating otherwise constitutively silent HOX genes. The finding that NCs can directly participate in the repair of experimental cartilage defects in goats, combined with the early observations of safety and feasibility in human, opens the clinical perspective of using nonhomotopic chondrocytes for enhanced cell-based articular cartilage repair.

RESULTS

HOX expression profile of human NCs

Comparative qualitative analysis of the whole HOX gene network in NCs and ACs by duplex polymerase chain reaction (PCR) showed several differentially expressed genes (Fig. 1A). Specific genes in the loci HOXC and HOXD were consistently expressed only by ACs. Quantitative reverse transcription PCR (qRT-PCR) confirmed expression of HOXc4, HOXc5, HOXc8, HOXD3, and HOXD8 in ACs, and only at baseline or undetectable levels in NCs (Fig. 1B). The differential gene expression was observed in chondrocytes from native human cartilage and following dedifferentiation or subsequent chondrogenic redifferentiation in vitro, thus establishing a set of markers constitutively distinguishing NCs from ACs (Fig. 1B), independent of the differentiation stage. The expression pattern of the identified HOX genes was assessed in other cartilage types from neuroectodermal (ear cartilage) and mesodermal (ankle cartilage) germ layers (Fig. 1C), confirming a consistent association with the tissue developmental origin. The findings thus outline a general possibility to distinguish neuroectoderm- from mesoderm-derived chondrocytes.

Comparison of NCs and ACs with mesenchymal stromal/stem cells from human bone marrow (BMSCs, mesoderm-derived) or from human dental pulp (DPCs, neuroectoderm-derived) indicated that the HOX expression pattern is more similar in cells of a common embryologic origin (NCs and DPCs versus ACs and BMSCs) than in cells with a common phenotype (for example, cells from hyaline cartilage such as NCs and ACs versus mesenchymal progenitor cells such as DPCs and BMSCs) (fig. S1). Furthermore, chondrogenic differentiation of BMSCs did not alter the activation of HOX genes (fig. S1). Our data confirm that HOX profiles capture developmental-related molecular identity and positional memory also in adult cells (19). Thus, they are well suited for studying environment-driven NC plasticity following heterotopic transplantation.

Self-renewal capacity of human NCs

We next assessed whether HOX expression profiles are associated with features of self-renewal, here defined [according to assays developed

Fig. 1. HOX expression profile of human NCs. (A) Duplex PCR of the whole HOX gene network in human ACs and NCs from five donors after monolayer expansion. Actively expressed genes are depicted in gray, whereas genes whose expressions were under the limits of detection are shown in white. HOXA, HOXB, HOXC, and HOXD describe the four HOX clusters; each of the clusters is located on one chromosome and, together with the following number, builds up the gene name. For donors 1, 2, and 3, NCs and ACs were harvested from the same individual.

(B) Real-time qRT-PCR analysis of HOX genes in ACs and NCs at different stages of differentiation. (C) Real-time qRT-PCR analysis of HOX genes in neural crest–derived ear and mesodermal ankle chondrocytes. Data in (B) and (C) were normalized to GAPDH. Data in (B) and (C) are means ± SD; n is the number of cartilage donors. All differences between cell sources in corresponding conditions were significant. For each donor, two experimental replicates were generated and analyzed; n.d., below limit of detection.
for other mesenchymal cell systems (20) as the capacity to generate differentiated, functional progenies following serial cycles of cloning. Freshly isolated human NCs and ACs were clonally expanded, with associated phenotypic dedifferentiation, and subsequently redifferentiated by 3D culture in chondrogenic medium. The resulting engineered cartilaginous tissues were digested to generate new clonal strains of de-differentiated cells (subcloning) (Fig. 2A) for ultimate assessment of their in vitro and in vivo chondrogenic ability. Compared to ACs, primary NCs contained a higher number of clonogenic cells (37% versus 21%; $P < 0.05$, Bayesian statistical modeling) (fig. S2A), and these NC-derived clones had a significantly faster proliferation rate than AC-derived clones (fig. S2B).

After the first cloning, similar percentages of NC and AC clones were capable of chondrogenic or osteogenic differentiation (Fig. 2B), as assessed by the generation of cartilaginous tissues or the deposition of mineralized matrix in vitro (fig. S2C). The percentage of AC-derived chondrogenic and osteogenic clones (40 and 30%, respectively) was in the range of the expected donor-related variability and consistent with a previous publication reporting 60% chondrogenic and 25% osteogenic clones within human AC populations (21). The onset of mineralization/osteogenic differentiation (fig. S2C) was associated with a 17.9 ± 3.6-fold increase (mean ± SD) of the master osteogenic transcription factor CBFA-1 (22) as compared to nonmineralizing/nonosteogenic clones [$n = 4$ clonal populations per group; $P < 0.05$ by one-way analysis of variance (ANOVA)]. The frequencies of osteochondrogenic clones (that is, populations with the ability of both osteogenic and chondrogenic differentiation) and of clones that formed cartilage in vivo were respectively 1.8- and 3.3-fold higher for NCs than for ACs (Fig. 2, B and C).

After subcloning, none of the AC strains maintained a chondrogenic capacity, whereas some NC strains formed cartilage tissues in vitro (23%) and in vivo (60%) (Fig. 2, B and C). The osteogenic differentiation capacity decreased in both NC and AC subclones compared to clones, such that the percentage of osteogenic subclones was up to fivefold lower and no osteochondrogenic subclone could be identified (Fig. 2B and fig. S3). These findings challenge the recently claimed multipotency of NCs (23).

**Environmental plasticity of human NCs in mice**

We then investigated whether NCs display features of environmental plasticity, here defined by analogy with developmental models (15) as the ability to adopt the Hox expression profile of the recipient site. We thus implanted human NCs in the form of engineered cartilaginous

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**Fig. 2.** Self-renewal capacity of NCs. (A) Serial clonal analysis. Cartilaginous tissue was engineered from single-cell clones derived from human nasal septum or articular cartilage (cloning) and digested for the generation of new clones (subcloning). Tests were conducted in vitro and in vivo in mice. (B) Summary of osteogenic (O), chondrogenic (C), and osteochondrogenic (OC) differentiation capacity of clones and subclones in vitro. (C) Quality of cartilage formation by NC and AC clones and subclones after 6 weeks ectopically in vivo, quantified using the Bern score (53). Score of 0 to 3: nonchondrogenic; score of 3.1 to 9: chondrogenic, with at least some areas positively stained for Safranin O; representative nonchondrogenic and chondrogenic clone and subclone are shown. Percentages refer to the total number of clones or subclones out of $n = 10$ and $28$ for AC clones and subclones, respectively, and $n = 27$ and $35$ for NC clones and subclones, respectively.
constructs into subcutaneous pockets of nude mice—a site of mesodermal origin and here verified to include Hox-positive cells (fig. S4). After 5 weeks, the explanted cells were identified to be of human origin by Alu in situ hybridization (Fig. 3A). Using human-specific primers, we demonstrated that HOX genes, which were silent or only minorly expressed in in vitro–cultured NCs (that is, HOXC4, HOXC5, and HOXD8), were up-regulated to levels similar to native articular cartilage (Fig. 1B) upon in vivo implantation (Fig. 3B). Induction of HOXC4 and HOXD8 expression at the protein level (Fig. 3C) confirmed the HOX reprogramming capacity of NCs. The in vivo–activated HOX genes remained expressed even after subsequent long-term (42 days) culture of the explanted construct in complete medium, indicating the stability of the acquired changes (Fig. 3B). HOX8 and HOXD3 were not activated upon in vivo implantation, which could be attributed to sitespecific conditions regulating the pattern of induced HOX genes.

Environmental plasticity of autologous goat NCs in articular defects

The environmental plasticity of NCs and their potential compatibility at an articular cartilage site were further assessed in a large-animal (goat) study. Experimental defects in a typical model (trochlear compartment) were filled with grafts based on green fluorescent protein (GFP)–transduced autologous NCs (Fig. 4A). We first verified that the Hox gene expression pattern in goat cartilage is similar to human, with Hoxc4, Hoxc5, Hoxc8, and Hoxd3 being expressed in goat ACs (gACs) but not in goat NCs (gNCs) (Fig. 4B). Four weeks after transplantation into an articular knee defect, GFP-positive gNCs were identified in regions of the repair tissue that stained positive for proteoglycans as well as in surrounding fibrous tissue (Fig. 4C), indicating their survival and suggesting contribution to the repair process. In situ colocalization of Hoxc4 and GFP expression (Fig. 4D) demonstrated the ability of gNCs to modify the memory of their biological origin and to adopt the Hox-positive profile of the implantation site.

Human HOX gene expression regulation in vitro

Toward identifying a potential mechanism involved in the HOX gene profile switch, human NCs were cultured under conditions mimicking different features of the natural joint environment. Hox gene expression in NCs was not induced by different applied factors (fig. S5), including a synovial fluid component (hyaluronic acid); inflammatory cytokines typically produced by ACs (interleukin-1β and tumor necrosis factor–α); molecules that have been described to activate HOX expression in human embryonic cells [retinoic acid (24) and cyclic adenosine 3′,5′-monophosphate (25)]; a key molecule involved in articular cartilage development (growth and differentiation factor 5) (26, 27); and soluble factors secreted by ACs, as tested by coculture in physically separated transwell systems or by application of AC-conditioned medium. The investigated HOX genes were also not activated by NC cultivation on a scaffold prepared with the extracellular matrix of articular cartilage (28) or under a regimen of mechanical conditioning resembling compressive deformation during joint loading (29) (fig. S5).

Instead, GFP-transduced NCs cocultured in direct contact with ACs or other Hox-positive mesodermal cells, such as synovial membrane fibroblasts, changed the expression profile for HOXc4, HOXd3, and HOXd8 (Fig. 5A and fig. S6A). This was confirmed representatively for HOXc4 by in situ hybridization in GFP-positive NCs (Fig. 5, B and C). The fact that HOXD3 was activated in NCs by coculture with ACs, in contrast to coculture with synovial membrane fibroblasts or in vivo subcutaneous implantation, and that HOXc5 was only activated in vivo suggests a different role for specific environmental parameters in selectively regulating the HOX gene network. When NCs were cultured together with formalin-fixed ACs, induction of HOX genes
was blocked (fig. S6B). This suggests that HOX-positive ACs trigger the expression of HOX genes either by paracrine signals acting only over a short distance or by membrane protein clustering/movement (29).

**NCs for repair of articular cartilage defects in goats**

A longer-term study in goats was performed to obtain preclinical evidence of gNCs for the repair of articular cartilage defects. Tissue-engineered constructs were generated using autologous gNCs and gACs (serving as controls) and implanted into experimental defects created at a clinically relevant location, namely load-bearing sites of the articular condyle (Fig. 6A). Per the semiquantitative O’Driscoll scoring system (30) (table S1), the quality of the repair tissue significantly improved from 3 to 6 months after implantation only when using gNCs [from 10.1 ± 1.5 at 3 months (n = 4) to 15.7 ± 1.7 after 6 months (n = 5); means ± SEM], such that at 6 months the repair quality achieved by gNCs was statistically superior to gAC controls (11.3 ± 1.8, mean ± SEM) (Fig. 6B and table S2). The improved quality of the repair tissue using gNCs compared with gACs was histologically confirmed by a stronger and more uniform staining for glycosaminoglycans at 6 months (Fig. 6C).

**Pilot NC implantation in human articular cartilage lesions**

To address the safety and feasibility of autologous NCs for the clinical treatment of posttraumatic full-thickness cartilage defects in the knee joint, in October 2012, we started recruiting patients in a pilot trial (http://clinicaltrials.gov identifier: NCT01605201; status: 7th patient treated). In the so far treated patients, followed up to 18 months after implantation, no systemic or local adverse events were observed (table S3), thus providing preliminary evidence of the safety and feasibility of the procedure. Magnetic resonance imaging (MRI) of the first patient before and 4 months after surgery indicated filling of the defect and no graft delamination, with strong reduction of subchondral bone edema (Fig. 7). The complete results of the still ongoing clinical trial will be the subject of a separate report.

**DISCUSSION**

Our findings demonstrate that neural crest–derived, HOX-negative, differentiated cells from human adult nasal cartilage, similar to what is currently known for embryonic developmental models (12) or stem cell systems (25, 31), exhibit features of self-renewal and environmental plasticity. These properties were respectively defined as the tissue regenerative capacity following serial cloning and the acquisition of a HOX pattern similar to the one of the recipient site upon transplantation. The principle was exploited for the preclinical and pilot clinical translation of autologous NCs for the unmet need of articular cartilage repair.

We initially identified a set of HOX genes capturing the different ontogeny of NCs and ACs, which form biochemically similar tissues but derive respectively from the neuroectoderm and the mesoderm. The same markers could distinguish ear from ankle chondrocytes, as well as DPCs from BMSCs. The data indicate that chondrocytes retain a positional signature inherited by the embryological origin (32–34), as proposed for epithelial (19), hematopoietic (18), and stromal (35) cells. The finding represents the basis for fundamental studies on the stability of NC molecular identity, on the assessment of reprogramming by environmental cues, as previously reported for embryonic mammalian limb bud (36) or thymic rat epithelial cells (37), and on the compatibility of their regenerative programs in heterotopic transplantation models, similar to what has been proposed for skeletal progenitors (16, 35).
As compared to HOX-positive ACs, the identified HOX-negative expression profile of human NCs was associated with a higher self-renewal and regenerative capacity, here assessed by the potential of forming cartilage tissues following extensive expansion (>45 population doublings) across cycles of clonal dedifferentiation and redifferentiation [more than three orders of magnitude changes in type II collagen mRNA (21)]. These observations are consistent with previous reports on the close link between Hox-dependent pathways and the self-renewal program of hematopoietic stem cells in physiology and pathology (38). Future studies will be required to identify the factors activating HOX gene expression, as well as to investigate whether the lack of specific HOX genes, such as HOXC4, is merely associated with or has a direct functional role in the self-renewal/plasticity of NCs. Assessment of the functional importance of expressed or silent HOX genes, however, will be challenging because complex interactions between the HOX proteins, their cofactors, and multiple other genes are expected to regulate the translation of HOX signaling into cellular function [see review (10)].

Implantation of autologous, GFP-labeled NCs in experimental articular cartilage defects in goats allowed to demonstrate (i) their direct contribution to the formation of the repair tissue, similar to what was

Fig. 5. Regulation of human HOX genes after coculture of NCs with ACs. (A) Real-time qRT-PCR analysis of HOX genes in human NCs after coculture with ACs for 7 days. Mixed cocultures were separated into the initial GFP-positive NC population and GFP-negative AC population by fluorescence-activated cell sorting (FACS). Separately expanded cells that were never cocultured served as controls. Data are means ± SD (n = 7 experimental runs with cells from different donors). (B) In situ colocalization of HOXC4 mRNA (red) and GFP protein (green) in GFP-labeled NCs when cocultured with ACs. The lower image shows a magnified view of the dotted region. Scale bars, 40 μm. (C) Separately cultured AC controls express HOXC4 mRNA (red), whereas NC controls express GFP, but not HOXC4. Cell nuclei were stained with 4',6-diamidino-2-phenylindole. Scale bars, 40 μm.

Fig. 6. Goat NCs in articular cartilage repair. (A) Two AC and two NC constructs per goat were implanted into condylar defects of a total of six goats and harvested after 3 (n = 3 goats) and 6 months (n = 3 goats). The diagram indicates the cases of initial construct delamination, with consequent elimination from the analysis and thus a reduction of the total number of scored explants as indicated. These delamination cases, merely related to the surgical challenges of the model, had an identical incidence for gAC- and gNC-based grafts. (B) O’Driscoll scores (30) of the repair quality of the gNC- or gAC-treated goat articular defects at 3 and 6 months (n = 3 animals per time point) after implantation. Data are means ± SEM (n = 4 and n = 5 scored replicates per group at 3 and 6 months, respectively). The indicated P values were calculated by Student’s t tests. (C) Safranin O and Alcian blue staining of representative repair tissues at the defect site (d) and of adjacent native articular cartilage (nac) 6 months after implantation of gNCs or gACs. The lower Alcian blue images show higher magnification (scale bar, 50 μm) of the regions framed in the respective upper panels (scale bar, 1 mm). Images are representative of n = 5 replicates.
implantation introduced more than 20 years ago in a clinical setting (1), our study differs not only in the used cell source, namely, NCs instead of ACs, but also in the grafting of a fully developed cartilage tissue as opposed to delivering cells by a gel or scaffold material. A tissue therapy rather than cell therapy for cartilage repair is expected to facilitate surgical handling and postoperative loading of the graft, as well as to protect implanted cells by the inflammatory factors at the implantation site, thanks to the deposited extracellular matrix (46). The tissue therapy concept is directly linked to the use of NCs because these cells, unlike ACs, allow more reproducible engineering of higher-quality cartilaginous grafts (5–7). The nasal biopsy necessitates a third operation in addition to the diagnostic arthroscopy that has to be performed in most cases to confirm the indication for a cellular therapy and during which ACs can be directly harvested. However, such operation can be performed under local anesthesia, is associated with minimal donor site morbidity (47), and avoids creating an additional damage to the already affected joint, shown to be potentially detrimental to the surrounding healthy articular cartilage (48).

Early assessments of adverse events and of maintenance in place of the repair tissue warrant proceeding with a larger cohort of patients, a longer-term follow-up, and the introduction of efficacy-related outcome parameters. Noteworthy, engineered cartilage based on autologous NCs has been recently reported to support safe and functional reconstruction of the nasal alar lobule in five patients, further underlining the regenerative capacity of the cell source (47). In parallel with carrying out further clinical studies, future adoption of NC-based engineered tissues for articular cartilage repair will require developing innovative manufacturing paradigms to address the standardization, scalability, and ultimately cost-effectiveness of the treatment (49).

MATERIALS AND METHODS

Study design

Preclinical study design. The objective of our study was to determine the self-renewal capacity and environmental plasticity of human neural crest–derived NCs and to demonstrate their compatibility and preclinical effectiveness for articular cartilage repair. Self-renewal capacity was demonstrated after serial cloning by the ability of cartilage formation in vitro and ectopically in vivo. The environmental plasticity of NCs was monitored by the cells’ ability to adapt the molecular HOX expression profile to that of the subcutaneous (human NCs in mice) or articular cartilage (autologous goat cells) environment. Cartilage biopsies from a total of n = 14 human donors were used to compensate for a known interindividual variability. The specific number of biological replicates (=donors) used for each experiment is indicated in the figure legends. The preclinical effectiveness of NCs for articular cartilage repair was tested in a goat model, where tissue-engineered grafts generated by gNCs or gACs were implanted into articular defects of 6 mm in diameter, and tissue repair was assessed histologically 3 and 6 months after implantation. The numbers of animals and assessed replicates are indicated in the figure legends.

Clinical study design. A phase 1 clinical trial was initiated to test the safety and feasibility of using tissue-engineered autologous nasal cartilage for the regeneration of articular cartilage in the knee after traumatic injury (http://clinicaltrials.gov Identifier: NCT01605201). Inclusion criteria for a total of 10 patients were full-thickness cartilage lesions [from 2 to 8 cm², ICRS (International Cartilage Repair Society)]
grade III to IV] on the femoral condyle and/or trochlea of the knee and age up to 55 years. Exclusion criteria were other chronic knee pathologies, previous major surgeries of the knee, or other conditions known to compromise cartilage repair. The primary outcomes for the trial were the incidence of systemic or local adverse events or reactions (including allergic reactions, wound infections, joint infections, and complications at the nasal biopsy harvest site) and the maintenance in place of the graft or of the repair tissue, as assessed by MRI up to 24 months after surgery.

**Regulatory compliance for human and animal studies**

All human samples were collected with informed consent of the involved individuals. All animal experiments were performed in accordance with Swiss law, after approval by the responsible veterinary offices of Bern (KantonaIes Veterinäramt Bern) and Zürich (KantonaIes Veterinäramt Zürich). Human studies were approved by the cantonal ethical authority of Basel [EKNZ (Ethikkommission Nordwest- und Zentralschweiz)] and by the Swiss regulatory agency for therapeutic products (Swissmedic).

**Chondrocyte isolation, cultivation, and differentiation**

ACs and NCs were isolated postmortem from articular knee joints of healthy human condyles and tibia plateau and from nasal septum, respectively. Auricular and ankle cartilages were isolated respectively from the ear and ankle joint. Chondrocyte expansion and differentiation under various conditions is described in Supplementary Methods.

**Ectopic implantation of human chondrocytes in vivo**

For in vivo investigations, $3 \times 10^6$ expanded human chondrocytes were seeded onto a collagen type I/III scaffold (6 mm in diameter) (Chondro-Gide, Geistlich Pharma AG) and cultured for 1 week in differentiation medium before implantation into subcutaneous pouches of nude mice. For each donor ($n = 6$ donors), four to eight constructs were generated and implanted in mice (two replicates implanted per mouse). Constructs were harvested after 5 weeks in vivo and assessed histologically and for gene expression. For two donors, constructs were kept for another 2 or 6 weeks in complete medium. In vitro control constructs were kept in differentiating medium for 1 week followed by 5 weeks in complete medium.

**Autologous gNC constructs in articular cartilage defects**

To investigate the environmental plasticity of NCs and their compatibility with articular joints, a 6-mm circular biopsy of nasal cartilage was harvested from two adult female goats after unilateral incision of the mucosa of the nasal septum. The isolated gNCs were expanded in the presence of fibroblast growth factor 2 (5 ng/ml) as previously described (50) before transduction with a GFP lentivirus at a multiplicity of infection of 10 and seeding ($4 \times 10^4$ gNCs/mm$^2$) on a Chondro-Gide membrane (Geistlich Pharma AG). FACS data acquisition and analysis were performed with CellQuest Pro software (Becton Dickinson). After 2 weeks of chondrogenic culture in vitro, four autologous NC-based constructs per animal ($n = 2$ animals) were implanted in four articular defects (6 mm in diameter and 1 mm in depth) generated in the same trochlea (Fig. 4A). Constructs were fixed to the surrounding articular cartilage with four stitches. Goats were sacrificed 4 weeks later, and samples were harvested for immunohistochemical and histological analyses.

To investigate the preclinical effectiveness of gNCs for articular cartilage repair, surgical procedures were performed for six adult female goats as described above with the following modifications: (i) four circular biopsies of 6 mm were additionally harvested from the articular caprine condyle; (ii) cartilaginous tissues were generated with autologous gNCs and gACs; (iii) two gNC- and two gAC-based constructs per animal were unilaterally implanted into the same condyle and followed up for 3 ($n = 3$ animals) and 6 months ($n = 3$ animals) (Fig. 6A). Repair tissues were analyzed by Safranin O and Alcian blue staining as described in the histology section and quantified according to the O’Driscoll score (30). In particular, mean scores from three independent observers were considered for each parameter and location (that is, medial and lateral regions adjacent to the native cartilage and central part of the defect).

**Gene expression analysis**

RNA isolation and qualitative duplex PCR were performed as described previously (51). Complementary DNA quantification was performed in duplicates by qRT-PCR using 7300 Real Time PCR Systems (Applied Biosystems) and normalized against GAPDH expression as described earlier (52). All analyzed genes are listed in table S4. The primers used to identify gene expression of the key markers ($HOXC4$, $HOXC8$, $HOXD3$, and $HOXD8$) were confirmed to be human-specific using subcutaneous (mesodermal) mouse tissue as a control, where the primers failed to amplify the same mouse Hox genes. Mouse-specific primers for Hox4, Hox5, Hox3, and Hox8 were used to investigate the expression of Hox genes in murine subcutaneous tissue (table S4). Colocalization of HOXC4 mRNA with GFP mRNA or the GFP protein was detected in situ with QuantiGene ViewRNA ISH Cell Assay or QuantiGene ViewRNA ISH Tissue Assay following the manufacturer’s (Affymetrix) instructions. GFP mRNA was labeled with Cy3 (excitation 554 nm/emission 576 nm) and false-colored in green to coincide with the GFP color. HOXC4 mRNA was labeled with Cy5 (excitation 644 nm/emission 669 nm) and false-colored to red.

**Clinical trial surgery**

To harvest the nasal cartilage, the mucous tissue on the nasal septum was incised and lifted to punch a biopsy (6 mm in diameter) out of the anterior part of the underlying nasal septal cartilage. NCs were isolated and expanded as described above, using autologous serum instead of fetal bovine serum. Cells were then seeded ($4 \times 10^4$ NCs/mm$^2$) and cultured in a collagen sponge (Chondro-Gide; 30 $\times$ 40 mm, 1.5 mm thick) in the context of a quality management system and Good Manufacturing Practice facility established within the University Hospital Basel. Four weeks after the harvesting of the autologous nasal cartilage biopsy, the damaged cartilage tissue was removed by mini-arthroscopy, and the defect was debrided down to the subchondral bone to create a stable rim of healthy cartilage. The tissue-engineered nasal cartilage autograft was trimmed to the defect size and placed into the defect. The graft was then secured to the surrounding tissue with resorbable polyfilament suture material (Vicryl, Ethicon) and fibrin adhesive (Tisseel, Baxter), and the arthroscopy was closed layer by layer. MRI of the defect site was performed using a $3T$ magnetic resonance imager (Verio, Siemens Medical Solutions) with sagittal T2-weighted fast spin echo sequence (4630/91).

**Statistical analysis**

With GraphPad Prism 5 statistical analysis software, the proliferation rates of NC and AC clones ($n = 27$ and $n = 10$, respectively) and subclones ($n = 35$ and $n = 28$, respectively) were analyzed by one-way ANOVA applying the Kruskal-Wallis test (significant if $P < 0.05$). A nested two-way ANOVA was performed to investigate significant
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differences between native nasal (n = 4) and articular cartilages (n = 6), expanded NCs (n = 9) and ACs (n = 10), tissue-engineered cartilages generated by NCs (n = 10) or ACs (n = 11), and ear (n = 3) and ankle (n = 3) chondrocytes. Unpaired Student's t tests were applied to determine statistical significance of differences measured in O'Driscoll scores in the goat cartilage repair study. Bayesian analysis using the Markov chain Monte Carlo method was applied using the WinBug program to define statistical relevance in the cloning efficiency, which was considered significant when the modeling showed 95% credible interval above 0.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/251/251ra119/DC1 Methods

Fig. S1. HOX gene expression in human stromal cells from bone marrow and dental pulp.

Fig. S2. Clonogenicity and differentiation capacity of human NC and AC clones and subclones in vitro.

Fig. S3. Chondrogenic and osteogenic differentiation capacity of NC and AC clones in vitro.

Fig. S4. Expression of genes in subcutaneous murine tissue.

Fig. S5. Effect of different factors on HOX gene induction in human NCs.

Fig. S6. Co-culture of human nasal chondrocytes with synovial membrane fibroblasts or ACs.

Table S1. Parameters of cartilage repair quality according to O'Driscoll score.

Table S2. Scoring of the repair tissue in goats.

Table S3. Summary of treated patients.

Table S4. List of TaqMan gene expression assays from Applied Biosystems.

REFERENCES AND NOTES


27. F. Dell'Accio, J. Vanlaerwa, J. Bellemans, J. Neys, B. C. De, F. P. Luyten, Expanded phenotypically stable chondrocytes persist in the repair tissue and contribute to cartilage matrix formation.