

Chondrogenic Potential and Homogeneity of Cell Populations of Donor and Recipient Cells in a Fresh Osteochondral Allograft

A Case Report

Dominik R. Haudenschild, PhD, Eunmee Hong, PhD, Sandra Hatcher, MSc, CLSp (MB), and Amir A. Jamali, MD

Investigation performed at the Department of Orthopaedic Surgery, University of California Davis Medical Center, Sacramento, California

Fresh osteochondral allografts have been widely used to treat cartilage lesions for more than 100 years¹. Transplantation of cartilage and bone in the form of an allograft allows osseous healing while maintaining the articular cartilage architecture. This composite tissue transplant remains intact in vivo for extensive periods of time with a favorable mechanical and biological environment. The chondrocytes of the graft are thought to actively remodel the extracellular matrix environment, and thus contribute to the tissue integrity. We recently reported that allograft cells could survive up to twenty-nine years after transplantation without the need for systemic immunosuppression². Although mosaic cell populations have been demonstrated in other forms of transplantation, these have always been under the umbrella of long-term systemic immunosuppression^{3,4}. In a classic study, Langer and Gross⁵ showed that intact articular cartilage surfaces obtained by removing the subchondral bone of rat femoral heads and filling of the osseous segments with acrylic cement exhibited essentially no humoral immune response in contrast to that seen with minced cartilage or isolated chondrocyte transplants. This finding has been attributed to the so-called “immunoprivileged” status of articular cartilage, which protects the chondrocytes from the immune system of the host.

The extent to which allograft chondrocytes retain their gene expression profiles and chondrogenic capacities remains unknown. Our goal was to compare gene expression, proliferation rate, and chondrogenic potential between host and allograft chondrocytes isolated three years after an unsuccessful fresh osteochondral allograft transplant in the knee. The patient was informed that data concerning her case would be submitted for publication, and she provided consent. The study was performed in full compliance with our institutional review board (IRB). Our IRB at University of California Davis Medical Center does not consider case reports to be research and does not

expressly provide IRB approval. We have sought IRB approval for case reports on a number of occasions in the past and have been given written documentation of this policy.

Case Report

Tissue Source

A forty-eight-year-old woman with early osteoarthritis of the knee was treated with a fresh osteochondral allograft with use of cylindrical plugs prepared with commercially available instrumentation (Arthrex, Naples, Florida); the plugs were applied to the trochlea (20 × 20-mm plug), the medial femoral condyle (20 × 20-mm plug), and the lateral femoral condyle (15 × 15-mm plug). The graft was a whole fresh distal part of a femur (University of Miami Tissue Bank, Miami, Florida) from an eighteen-year-old male donor implanted twelve days after harvest. The tissue bank provided the allografts submerged in culture media (RPMI-1640 or lactated Ringer solution) with antibiotics at a refrigerated temperature (1°C to 10°C, never frozen). Transplants approximately 1.0 to 1.5 cm in diameter were created from the region of the allograft joint corresponding to the site of transplantation. Prior to implantation, bone marrow was removed from the transplant plugs with pulsatile irrigation, and the transplant was then plugged into the recipient site without any additional treatment.

The knee pain and osteoarthritis ultimately progressed, and a total knee arthroplasty was performed three years after the index procedure. Discarded tissues from the total knee arthroplasty were removed from the operative field, placed in sterile saline solution in the operating room, and processed for chondrocyte isolation within sixty minutes after surgery.

Cell Sex Determination by FISH to X and Y Chromosomal DNA

Fixed cells from cultured articular cartilage tissue were placed onto glass slides and dried, and dual-color fluorescence in situ

Disclosure: None of the authors received payments or services, either directly or indirectly (i.e., via his or her institution), from a third party in support of any aspect of this work. One or more of the authors, or his or her institution, has had a financial relationship, in the thirty-six months prior to submission of this work, with an entity in the biomedical arena that could be perceived to influence or have the potential to influence what is written in this work. No author has had any other relationships, or has engaged in any other activities, that could be perceived to influence or have the potential to influence what is written in this work. The complete **Disclosures of Potential Conflicts of Interest** submitted by authors are always provided with the online version of the article.

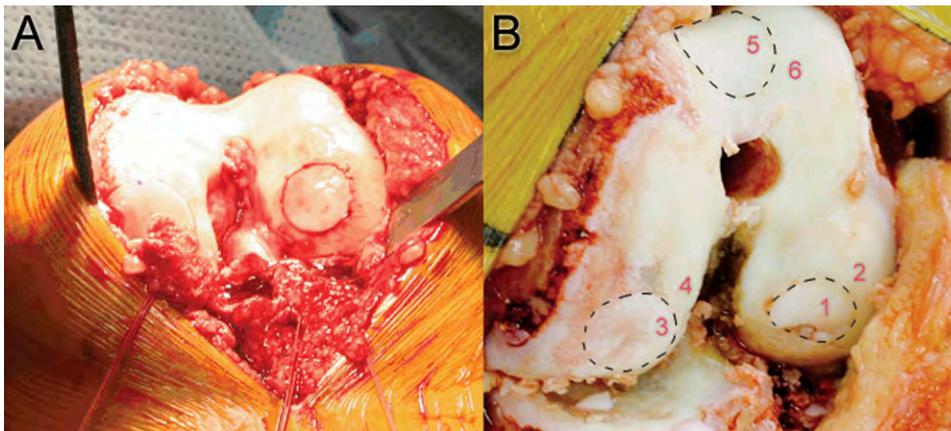


Fig. 1
Allograft at the time of implantation (**Fig. 1-A**) and three years after implantation at the time of total knee replacement surgery (**Fig. 1-B**). The numbers 1 through 6 indicate the sites of tissue harvest of allograft (odd numbers) and host cells (even numbers). The dotted lines and numbers were produced with an image-editing software program to illustrate the approximate site of the tissue harvested for cell isolation.

hybridization (FISH) was performed on the cells with the chromosome enumeration probes (CEPs) X SpectrumGreen and Y SpectrumOrange (Vysis, Des Plaines, Illinois). These probes are specific to the centromere region of Xp11.1-q11.1 and Yp11.1-q11.1 of the X and Y chromosomes. Female cells (XX) demonstrate two green signals (GG); male cells (XY) demonstrate one orange and one green signal (OG). The cells were denatured and hybridized overnight with use of a HYBrite system (Vysis). The slides were washed with 70% ethanol and then were dehydrated to 100% ethanol. Dried slides were counterstained with DAPI II (4',6-diamidino-2-phenylindole) (125 ng/mL). A total of 200 interphase nuclei were scored for each sample from donor sites 1, 3, and 5, as well as from host sites 2 and 6. Cells from site 4 were lost to contamination and were not assayed.

Chondrocyte Isolation and Expansion

The discarded tissues were rinsed with several changes of Hanks Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, California) containing 1% penicillin and streptomycin. Cartilage was dissected from three allograft sites and three corresponding adjacent host osteochondral sites (Figs. 1-A and 1-B). Cartilage from each site was removed from the subchondral bone with a sterile scalpel and minced into 1.5-mm fragments. Chondrocytes were released from the minced cartilage fragments by first rinsing them in 2.5% trypsin in Dulbecco Modified Eagle Medium (DMEM) for fifteen minutes at 37°C, followed by an overnight digestion in 2 mg/mL of type-IV bacterial collagenase (Sigma-Aldrich, St. Louis, Missouri) in DMEM with 2% calf serum and antibiotics in a shaking incubator set to 180 rpm and 37°C. The released cells were recovered by centrifugation at 1000 times gravity, rinsed once with DMEM, and counted with use of a hemocytometer. These cell populations were considered unpassaged (P0) and day 0 cells. For additional expansion, 1×10^5 cells were plated on tissue-culture-treated plastic and cultured for two weeks in DMEM containing 10% calf serum and antibiotics, with media changes every other day. To assess proliferation rates, cell numbers in subconfluent monolayer cultures were counted in a hemocytometer. Population doubling time (T_d) was determined for the culture with use of

Equation 1 (below), where N_0 is the number of seeded cells and N_1 is the number of harvested cells.

$$T_d = \frac{(\text{culture time})}{\log_2(N_1/N_0)} \quad \text{Equation 1.}$$

Pellet Culture Chondrogenesis Assays

The chondrogenic capacity of isolated primary chondrocytes (P0) was compared with that of passaged (P2) cells. Three-dimensional cell pellet cultures were established in serum-free chondrogenic medium. Briefly, 2.5×10^5 cells were placed in 15-mL conical polypropylene centrifuge tubes and pelleted by centrifugation at 150 times gravity for five minutes. Medium was gently replaced with 500 μ L of chondrogenic base medium (Lonza, Basel, Switzerland) containing 10 ng/mL of rhTGF- β 3 and an additional 300 ng/mL of rhBMP-7 (a gift from Dr. David Rueger, Stryker Biotech, Hopkinton, Massachusetts); the pellets were maintained in chondrogenic medium for up to fourteen days. The medium was changed every three to four days, and the growth factors were replenished with every medium change.

Gene Expression Analysis

RNA was isolated from 1×10^5 unpassaged (P0) host and allograft cells, and from seven-day pellet cultures with use of the RNeasy total RNA Kit (Qiagen, Valencia, California). cDNA was synthesized with use of the High Capacity Reverse Transcriptase Reagents, and real-time quantitative polymerase chain reaction (PCR) was performed with use of Assays-on-Demand TaqMan primers and probes and TaqMan reagents on an ABI Prism 7700 Sequence Detector (Applied BioSystems, Foster City, California). The TaqMan probe sets are shown in Table I. Assays were performed in triplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels with use of the recommended Δ Ct method.

Histological and Immunohistochemical Staining

Three-dimensional pellets of P2 cells were harvested after fourteen days of culture in chondrogenic media. Pellets were fixed in Bouin fixative and rinsed with 70% ethanol overnight. Samples were embedded in paraffin, and 5- μ m sections were cut. Sections

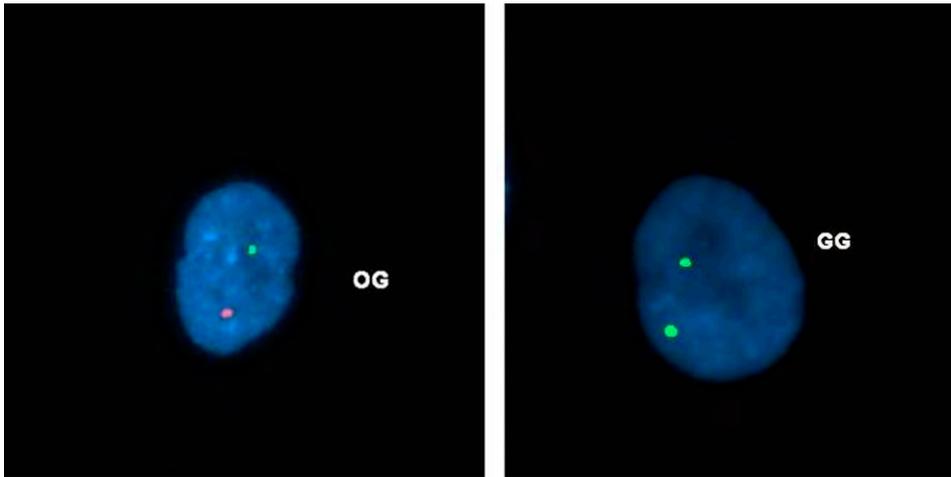


Fig. 2
Dual-color FISH results illustrating interphase nuclei with either XX or XY sex chromosomes. Cells were hybridized with the CEP X SpectrumGreen and CEP Y SpectrumOrange probes. Female cells show two green signals (GG); male cells show one green and one orange signal (OG) (1000× magnification).

were stained with hematoxylin and eosin and with 1% safranin-O to highlight cell morphology and sulfated glycosaminoglycans, respectively. Immunohistochemistry was performed with use of the antibodies shown in Table I. Commercially available antibodies were used at the recommended concentrations, and antibodies against cartilage oligomeric matrix protein (COMP) and a disintegrin-like and metalloprotease domain with thrombospondin motifs 7 (ADAMTS7) were generated in our laboratory and used as previously reported⁶⁷. Detection was with peroxidase-conjugated

secondary antibodies and 3,3'-diaminobenzidine (DAB) substrate according to the recommended protocols (ImmPRESS reagents and ImmPACT DAB; Vector Laboratories, Burlingame, California). Samples were counterstained with methyl green to identify cell nuclei for all sections except Sox9.

Source of Funding

This project was funded entirely by the Department of Orthopaedic Surgery, University of California Davis Medical Center.

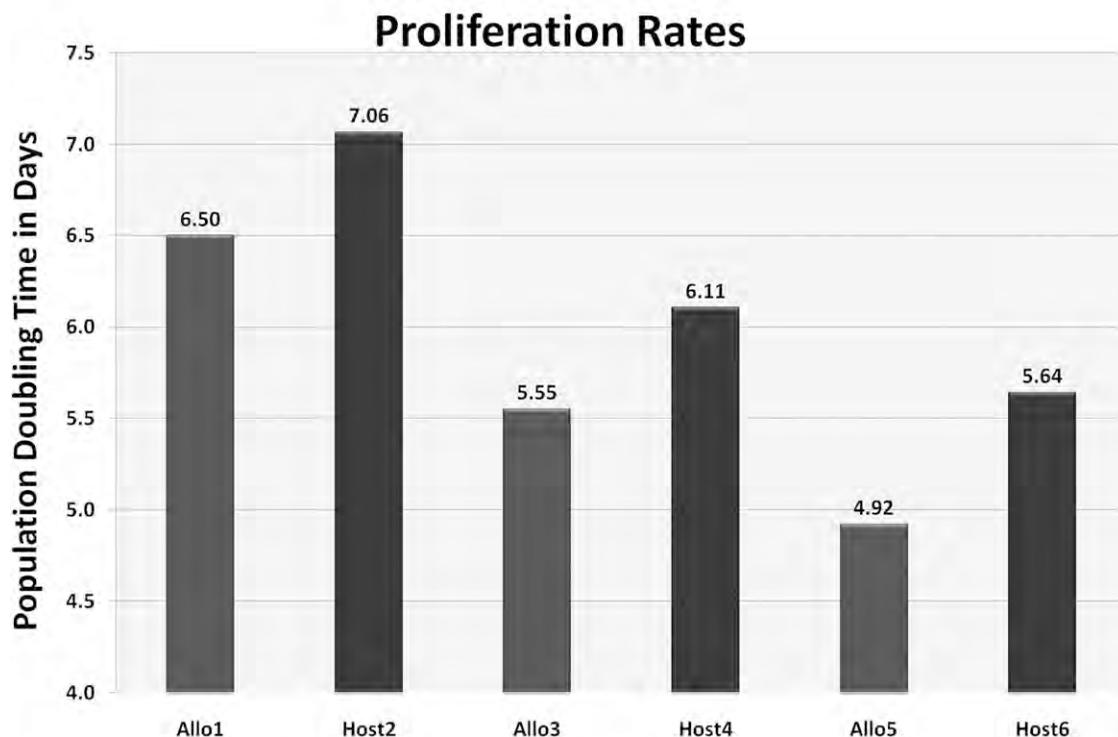


Fig. 3

The in vitro proliferation rate of isolated chondrocytes varied from region to region, but was consistently slower in cells isolated from the host tissue than in cells isolated from the adjacent allograft (on average, there was an $1.1\% \pm 3\%$ longer proliferation rate in cells from the host site compared with cells from the respective adjacent allograft).

Results

Cell Identification

The allograft cells, which came from a male donor, were transplanted into a female patient at the time of surgery. In order to determine whether the cells recovered from the three allograft sites and the adjacent host sites were of male or female origin, isolated chondrocytes were assayed for the presence of sequences specific for the X and Y chromosomes. A total of 200 cells were scored from each site, and, without exception, only male cells were recovered from the allografts, while only female cells were recovered from the adjacent host tissue (Fig. 2).

Cell Proliferation

All of the isolated chondrocyte strains from host and allograft sources showed robust proliferation in culture. The proliferation rate during monolayer expansion differed between cells isolated from the host and those isolated from allografts, and varied from region to region (Fig. 3). Cells from the host tissue required an average (and standard deviation) of $11\% \pm 3\%$ longer for population doubling than cells from the allograft tissue. There was also regional variation, with chondrocytes (from both host tissue and allograft) isolated from the trochlear groove proliferating the fastest. The average population doubling time of cells (host and allograft combined) from the condyles and the trochlear groove was 6.3 ± 0.6 days and 5.3 ± 0.5 days, respectively.

Gene Expression in P0 Chondrocytes

Gene expression was remarkably similar between cells from the host and allograft tissues at day 0 (Fig. 4). Extracellular matrix genes (aggrecan [Agc], Col2, and COMP) and the transcriptional regulator of chondrogenesis (Sox9) were all expressed at similar levels in chondrocytes isolated from host and allograft tissues. Matrix remodeling genes (ADAMTS7 and ADAMTS12) and a marker of hypertrophic differentiation (ColX) were also expressed at similar levels in chondrocytes isolated from host and allograft tissues. The exceptions were sites 1 and 2, in which there was somewhat greater variation between host and allograft in two of the eight genes tested—namely, the ColX level was higher in cells from allograft (site 1) than it was in cells from host (site 2) tissue, and ADAMTS12 showed the opposite trend. This is reflected in the larger error bars for these two genes (Fig. 4).

Chondrogenic Capacity

Chondrocyte strains from host and allograft tissues retained their chondrogenic capacity in an in vitro pellet culture assay, as measured by gene expression after seven days (Fig. 5). Gene expression profiles were very similar between pellets formed from host and those formed from allograft chondrocytes. There was high expression of matrix genes (Agc, Col2, and COMP) as well as the Sox9, and lower expression of ADAMTS7 and ADAMTS12, a gene expression profile consistent with chondrogenic differentiation of expanded cells. The exception

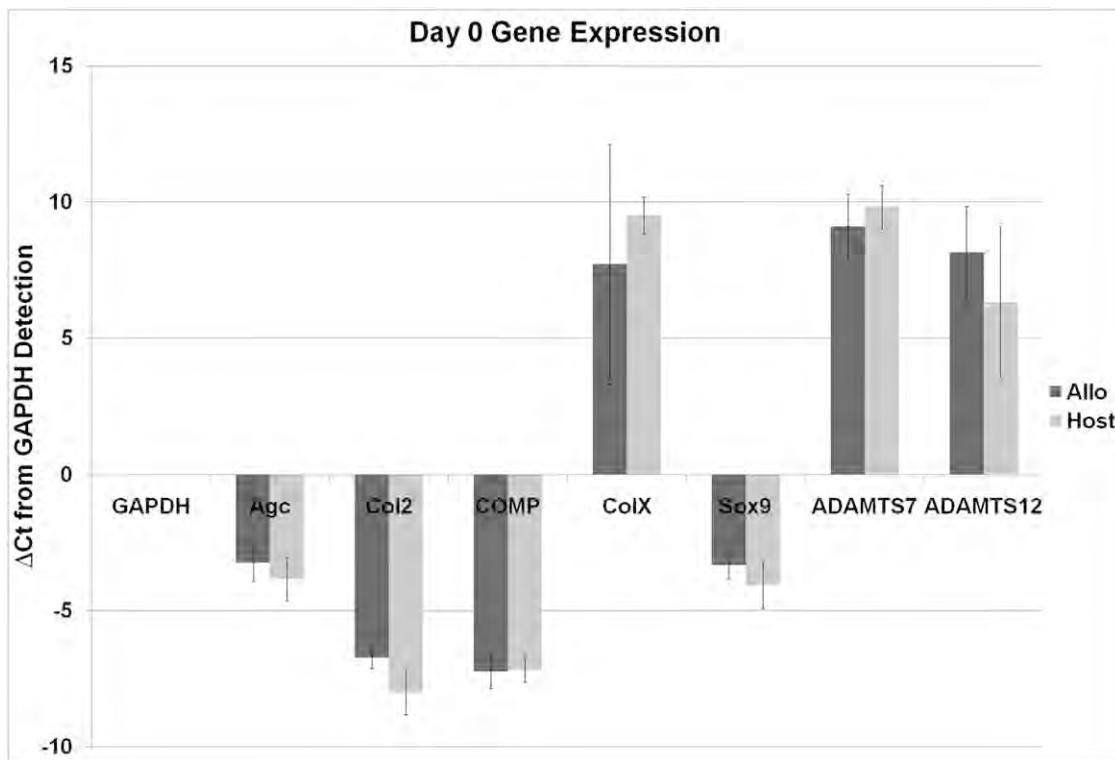


Fig. 4

Gene expression at day 0 is stable from region to region and between host and allograft as measured by the quantitative reverse transcription polymerase chain reaction (RT-PCR). The Y axis shows the average Δ Ct to GAPDH of the cells from the host (sites 2, 4, and 6) and the allograft (sites 1, 3, and 5). Negative values indicate the transcript was detected before GAPDH and, therefore, highly expressed. Error bars represent the standard deviation of the Δ Ct of the three sites.

TABLE I Summary of TaqMan Probe Sets for Real-Time Quantitative PCR*

Classification	Target	Antibody	Primer
Cartilage extracellular matrix proteins	Aggrecan	Chemicon AB1031	Hs00202971_m1
	COMP	Polyclonal rabbit anti-hCOMP (in-house)	Hs00164359_m1
	Type-II collagen	United States Biological C7510-19K	Hs01060345_m1
Marker of hypertrophy	Type-X collagen	N/A	Hs00166657_m1
Cartilage-specific transcription factor	Sox9	Santa Cruz H-90	Hs00165814_m1
Matrix remodeling enzymes	ADAMTS12	Santa Cruz E17	Hs00229594_m1
	ADAMTS7	Polyclonal rabbit anti-ADAMTS7	Hs00276223_m1
Housekeeping gene for normalization	GAPDH	N/A	4326317E

*PCR = polymerase chain reaction, COMP = cartilage oligomeric matrix protein, ADAMTS = a disintegrin-like and metalloprotease domain with thrombospondin motifs, and GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

was type-X collagen, which was more variable in pellets from allograft tissue than in pellets from host tissue sites. It was also noted that, despite the chondrogenic conditions used in the pellet cultures, the overall expression of matrix genes was lower than that in freshly isolated cells. Histochemical analysis of phenotype and matrix production after fourteen days in pellet culture revealed no apparent differences between cell pellets

from host sources and those from allograft sources. The amount and localization of safranin-O-stained proteoglycans and the extracellular matrix components aggrecan and type-II collagen were similar between the host and allograft cell pellets, as were the amounts and locations of proteases ADAMTS12 and ADAMTS7 as well as the chondrogenic transcription factor, Sox9 (Fig. 6).

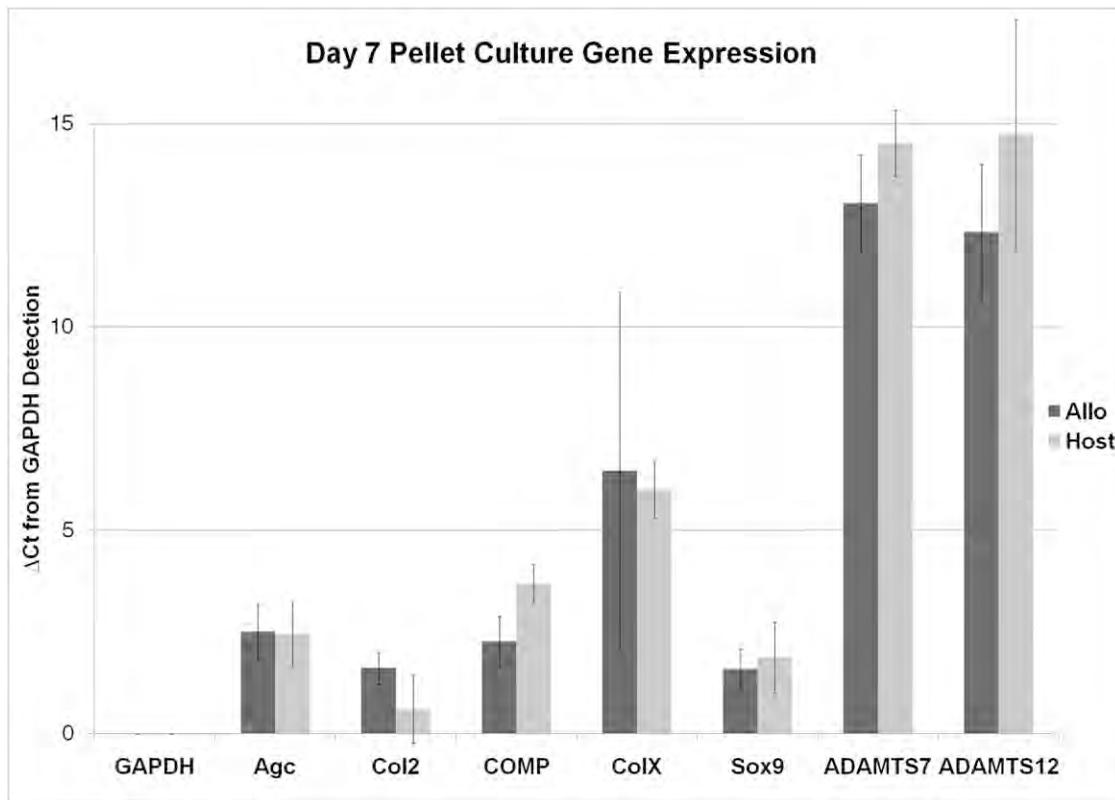


Fig. 5

Gene expression analysis at day 7 of chondrogenic growth in pellet culture. Gene expression is stable from region to region and between host and allograft as measured by the quantitative RT-PCR. The Y axis shows the average Δ Ct to GAPDH of the cells from the host (sites 2, 4, and 6) and the allograft (sites 1, 3, and 5). Error bars represent the standard deviation of the Δ Ct of the three sites.

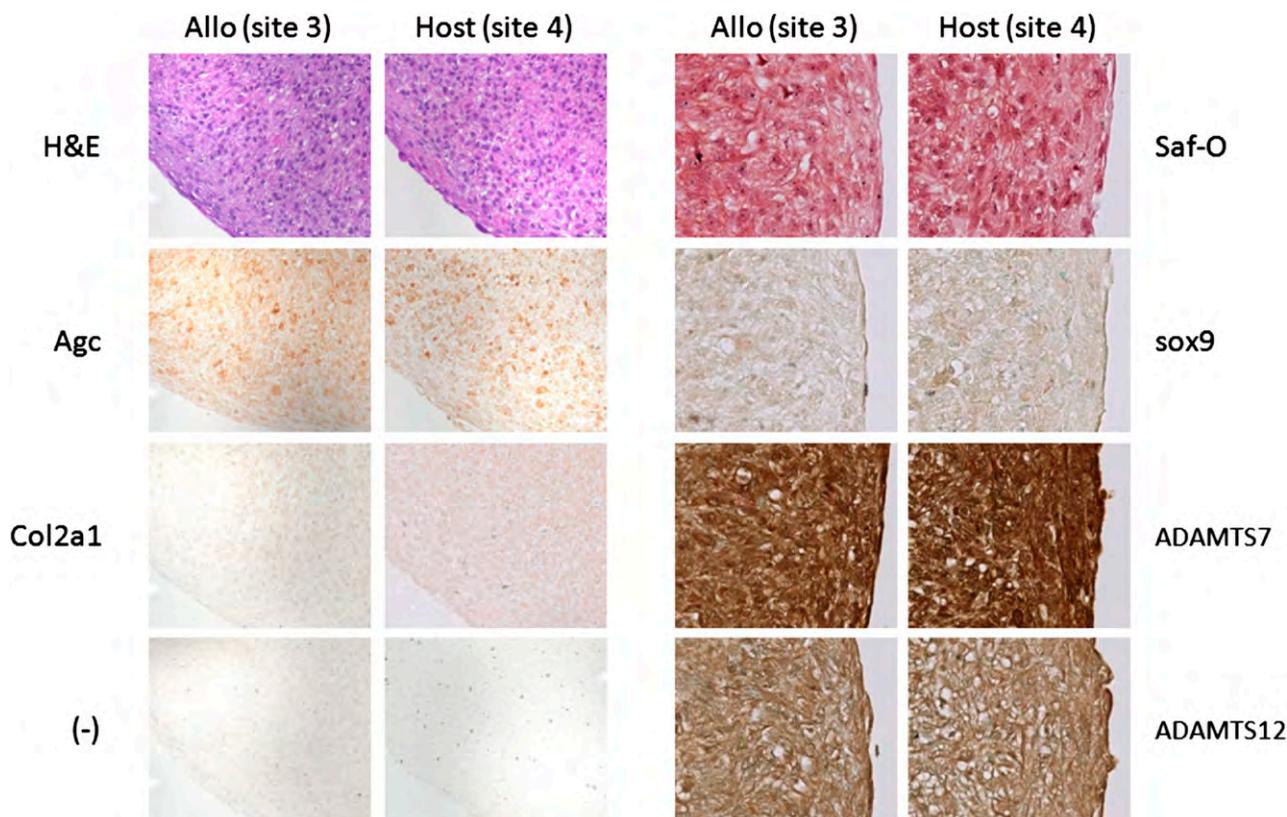


Fig. 6

Immunohistochemistry and histological appearance after fourteen days of chondrogenic growth in pellet cultures. The cell pellets were stained with hematoxylin and eosin (H&E), safranin-O (Saf-O), and various antibodies. The localization and amounts of aggrecan (Agc), type-II collagen (Col2a1), Sox9, ADAMTS7, and ADAMTS12 were essentially similar in the cell pellets formed from passaged chondrocytes isolated either from allograft or from adjacent host tissue. The (-) panel is representative of no primary antibody control.

Discussion

Osteochondral allografts are widely used to treat cartilage lesions. They have a long track record of success⁸⁻¹¹. Currently, they are particularly suited for use in large cartilage defects or those that involve the subchondral bone. Their clinical success has been widely attributed to their so-called “immunoprivileged” status, which protects the chondrocytes of the graft from the host’s immune system while the bone of the graft heals to the recipient¹². Despite their widespread use, little is known about the effects that transplantation has on the chondrocytes within the allograft or in the surrounding host cartilage tissue. Our patient presented a unique opportunity to study both the allograft and the surrounding host tissue three years after the original allograft procedure. The goals of this study were (1) to identify the homogeneity of cell populations in the graft and in the surrounding host cartilage regions and (2) to determine the levels of various chondrocyte markers in cells isolated from the allograft compared with the cells isolated from the surrounding host cartilage.

The cells isolated from the host and allograft tissues were assayed by FISH for the presence of X and Y chromosomes. Without exception, all of the allograft cells were male, and all of the host cells were female. This indicates that, although the cells remained viable and the tissues appeared well integrated

macroscopically, there was no cellular migration between the host and allograft tissues in the three years following the transplantation. This information is in concordance with the work of Langer and Gross⁵. More recently, Maury et al.¹³ showed histological evidence of chondrocyte survival in a fresh osteochondral allograft of the femoral condyle at twenty-five years after implantation. In spite of the evidence based on electron microscopy of the production of proteoglycans by these cells, their origin from the donor or recipient was not delineated. Furthermore, a comparison of the cellular machinery of the graft cells compared with the host cells was not carried out. Jamali et al.² reported on a fresh osteochondral allograft of the femoral condyle converted to total knee replacement twenty-nine years following transplantation. Since the graft had undergone degeneration, scrapings from the general area of the femoral condyle were sent for cytogenetic analysis. These revealed a mixed population of cells, including both those of the recipient (male) and those of the donor (female). Unfortunately, the scrapings could not be specifically linked to the host or the graft and most likely represented a combination of both tissues. Our patient had an unsuccessful allograft with a much shorter in vivo duration. Nevertheless, this short duration allowed us to closely scrutinize the areas of the knee at the time of total knee replacement and obtain “clean” samples

from the graft and from the host. This information provided additional support for the notion that cartilage is a closed system since, in this case, there was no evidence of chimerism of the chondrocytes between the graft and the host. Furthermore, we have shown that gene expression did not vary greatly between the host and graft tissue or, for the most part, between the allograft locations at the time of cell harvest, at day 7 of chondrogenic pellet culture, or after monolayer expansion (data not shown).

The proliferation rates did vary between cells isolated from the host and those isolated from the allograft tissues, with allograft cells having consistently shorter doubling times than cells isolated from immediately adjacent host tissues. With only one patient and one allograft donor, we can only speculate about the relevance of the differential proliferation rates between allograft and host tissues. One possibility is that the more rapidly dividing cells were isolated from a young donor, whereas the host patient was in her late forties. This would be consistent with previous findings that cells isolated from older patients proliferate at a reduced rate in monolayer culture¹⁴ and are less responsive to growth factor stimulation¹⁵.

Clinical Relevance

This study provides additional evidence of the clinical utility of fresh osteochondral allografts to treat cartilage defects.

In our case, chondrocytes from the allograft tissue remained as an isolated population with a high viability, and they retained their gene expression profiles and proliferation capacities. The gene expression and chondrogenic potential of allograft and host cells were essentially identical after three years in vivo. ■

Dominik R. Haudenschild, PhD
Eunmee Hong, PhD
Department of Orthopaedic Surgery,
University of California Davis Medical Center,
4860 Y Street, Suite 3800, Sacramento, CA 95817

Sandra Hatcher, MSc, CLSp (MB)
Department of Pathology and Laboratory Medicine,
University of California Davis Medical Center,
3740 Business Drive, Sacramento, CA 95820

Amir A. Jamali, MD
Joint Preservation Institute, 2825 J Street,
#440, Sacramento, CA 95816.
E-mail address: contactus@jointpreservationinstitute.com

References

1. Lexer E. Substitution of whole or half joints from freshly amputated extremities by free plastic operation. *Surg Gynecol Obstet.* 1908;6:601-7.
2. Jamali AA, Hatcher SL, You Z. Donor cell survival in a fresh osteochondral allograft at twenty-nine years. A case report. *J Bone Joint Surg Am.* 2007;89:166-9.
3. Starzl TE, Demetris AJ, Trucco M, Ricordi C, Ildstad S, Terasaki PI, Murase N, Kendall RS, Kocova M, Rudert WA, et al. Chimerism after liver transplantation for type IV glycogen storage disease and type 1 Gaucher's disease. *N Engl J Med.* 1993;328:745-9.
4. Yam PY, Petz LD, Knowlton RG, Wallace RB, Stock AD, de Lange G, Brown VA, Donis-Keller H, Blume KG. Use of DNA restriction fragment length polymorphisms to document marrow engraftment and mixed hematopoietic chimerism following bone marrow transplantation. *Transplantation.* 1987;43:399-407.
5. Langer F, Gross AE. Immunogenicity of allograft articular cartilage. *J Bone Joint Surg Am.* 1974;56:297-304.
6. Liu CJ, Kong W, Ilalov K, Yu S, Xu K, Prazak L, Fajardo M, Sehgal B, Di Cesare PE. ADAMTS-7: a metalloproteinase that directly binds to and degrades cartilage oligomeric matrix protein. *FASEB J.* 2006;20:988-90. Epub 2006 Apr 3.
7. Xu K, Zhang Y, Ilalov K, Carlson CS, Feng JQ, Di Cesare PE, Liu CJ. Cartilage oligomeric matrix protein associates with granulysin-epithelin precursor (GEP) and potentiates GEP-stimulated chondrocyte proliferation. *J Biol Chem.* 2007;282:11347-55. Epub 2007 Feb 15.
8. Aubin PP, Cheah HK, Davis AM, Gross AE. Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. *Clin Orthop Relat Res.* 2001;(391 Suppl):S318-27.
9. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D. Articular cartilage repair using allogeneic perichondrocyte-seeded biodegradable porous poly(lactic acid) (PLA): a tissue-engineering study. *J Biomed Mater Res.* 1995;29:1147-54.
10. Ghazavi MT, Pritzker KP, Davis AM, Gross AE. Fresh osteochondral allografts for post-traumatic osteochondral defects of the knee. *J Bone Joint Surg Br.* 1997;79:1008-13.
11. Shasha N, Krywulak S, Backstein D, Pressman A, Gross AE. Long-term follow-up of fresh tibial osteochondral allografts for failed tibial plateau fractures. *J Bone Joint Surg Am.* 2003;85 Suppl 2:33-9.
12. Williams SK, Amiel D, Ball ST, Allen RT, Tontz WL Jr, Emmerson BC, Badlani NM, Emery SC, Haghighi P, Bugbee WD. Analysis of cartilage tissue on a cellular level in fresh osteochondral allograft retrievals. *Am J Sports Med.* 2007;35:2022-32. Epub 2007 Aug 27.
13. Maury AC, Safir O, Heras FL, Pritzker KP, Gross AE. Twenty-five-year chondrocyte viability in fresh osteochondral allograft. A case report. *J Bone Joint Surg Am.* 2007;89:159-65.
14. Barbero A, Grogan S, Schäfer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis Cartilage.* 2004;12:476-84.
15. Guerne PA, Blanco F, Kaelin A, Desgeorges A, Lotz M. Growth factor responsiveness of human articular chondrocytes in aging and development. *Arthritis Rheum.* 1995;38:960-8.