http://dx.doi.org/10.2106/JBJS.L.01715


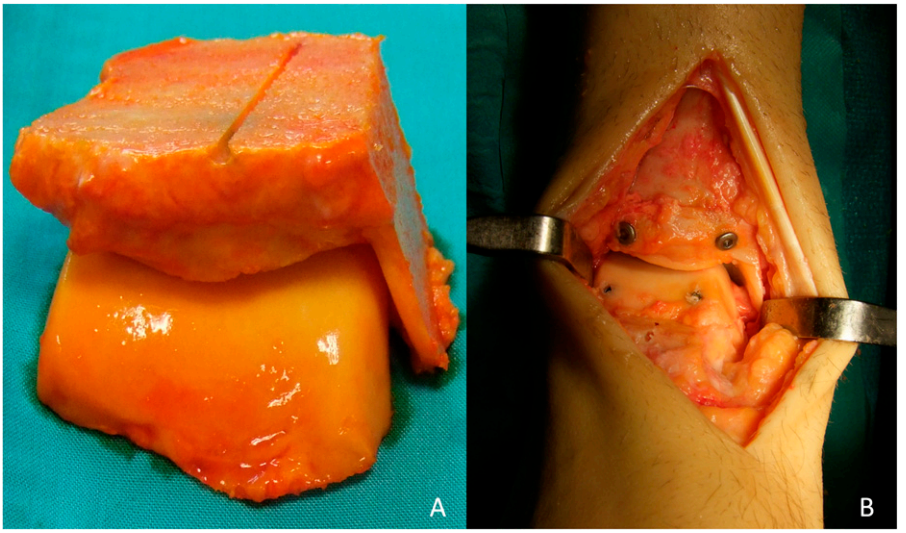


Fig. E-1

Photographs showing the surgical field of representative cases. **Fig. E1-A** Prepared bipolar fresh osteochondral allograft surfaces with an excellent macroscopic aspect of the articular cartilage. **Fig. E1-B** Implanted allograft using an anterior approach.

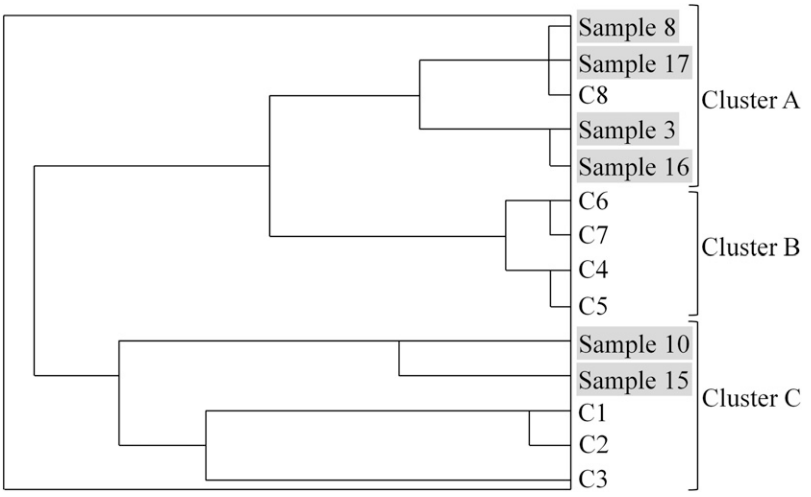


Fig. E-2

Dendrogram obtained by hierarchical clustering of six allograft samples (Cases 3, 8, 10, 15, 16, and 17) and eight defined control cell types: normal cartilage (C1 and C2), freshly isolated chondrocytes from normal cartilage (C3), cultured chondrocytes at P1 (C4) and P10 (C5), synovial fibroblasts at P3 (C6), freshly isolated osteoblasts (C7), and bone marrow-derived mesenchymal stem cells (C8). Three main clusters (A, B, C) were identified on the basis of collagen type-II, collagen type-IX, collagen type-X, aggrecan, SOX9, cathepsin B, MMP-13, alkaline phosphatase, RUNX2, osteocalcin, osteopontin, and bone sialoprotein gene expression levels.

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**TABLE E-1 Summary of Primer Sequences and Amplicon Size for Real-Time RT-PCR Analysis**

| Template | Primer Sequence | Amplicon Size (Base Pairs) |
|---|---|---|
| Collagen type II (Col II) | 5′-GACAATCTGGCTCCCAAC-3′ | 257 |
| | 5′-ACAGTCTTGCCCCACTTAC-3′ | |
| Collagen type IX (Col IX) | 5′-CAGGAAGAGGTCCCAAC-3′ | 175 |
| | 5′-GCTGGCTCACAGAAACC-3′ | |
| Collagen type X (Col X) | 5′-CCAGCACGCAGAATCC-3′ | 139 |
| | 5′-GTGTTGGGTAGTGGGC-3′ | |
| Aggrecan (Aggr) | 5′-GTCTCACTGCCCAACTAC-3′ | 157 |
| | 5′-GGAACACGATGCCTTTCAC-3′ | |
| SOX9 | 5′-GAGCAGACGCACATCTC-3′ | 118 |
| | 5′-CCTGGGATTGCCCCGA-3′ | |
| Cathepsin B (Cath B) | 5′-TGTGTATTCGGACTTCCTGCT-3′ | 113 |
| | 5′-GTGTGCCATTCTCCACTCC-3′ | |
| MMP-13 | 5′-TCACGATGGCATTGCT-3′ | 277 |
| | 5′-GCCGGTGTAGGTGTAGA-3′ | |
| Collagen type I (Col I) | 5′-GAGAGCATGACCGATGG-3′ | 251 |
| | 5′GTGACGCTGTAGGTGAA-3′ | |
| Alkaline phosphatase (AP) | 5′-GGAAGACACTCTGACCGT-3′ | 152 |
| | 5′-GCCCATTGCCATACAGGA-3′ | |
| RUNX2 | 5′-GGAATGCCTCTGCTGTTATG-3′ | 105 |
| | 5′-AGACGGTTATGGTCAAGGTG-3′ | |
| Osteocalcin (OC) | 5′-GCAGCGAGGTAGTGAAGA-3′ | 148 |
| | 5′-TCCTGAAAGCCGATGTGG-3′ | |
| Osteopontin (OP) | 5′-ATGATGGCCGAGGTGATAG-3′ | 119 |
| | 5′-GCTTTCCATGTGTGAGGTG-3′ | |
| Bone sialoprotein (BSP) | 5′-CAGTAGTGACTCATCCGAAG-3′ | 158 |
| | 5′-CATAGCCCAGTGTTGTAGCA-3′ | |
| GAPDH | 5′-TGGTATCGTGGAAGGACTCATGAC-3′ | 190 |
| | 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ | |

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**TABLE E-2 Allograft Genotyping***

| | Short Tandem Repeats | | | | | |
|---|---|---|---|---|---|---|
| | CD4 | | | VWA | | |
| Cases | R | A† | D | R | A† | D |
| 1 | 4-9† | 4-9† | 5-7 | 18-19† | 18-19† | 14-16 |
| 2 | 4-5† | 4-5† | — | 18-19† | 18-19† | — |
| 3 | 9-9‡ | 9-9‡ | 9-9‡ | 16-18† | 16-18† | 14-17 |
| 4 | 9-9† | 9-9† | — | 15-16† | 15-16† | — |
| 5 | 4-5‡ | 4-5‡ | 4-5‡ | 15-16† | 15-16† | 16-17 |
| 6 | 4-5† | 4-5† | 5-9 | 18-18† | 18-18† | 15-16 |
| 7 | 9-9† | 9-9† | 5-5 | 15-17† | 15-17† | 16-17 |
| 8 | 4-5† | 4-5† | 5-9 | 14-17† | 14-17† | 15-19 |
| 9 | 4-9† | 4-9† | 5-5 | 14-19† | 14-19† | 16-17 |
| 10 | 9-9† | 9-9† | 4-9 | 17-19† | 17-19† | 17-18 |
| 11 | 4-5 | 5-9† | —† | 15-17 | 15-18† | —† |
| 12 | 9-9 | 5-5† | 5-5† | 16-18 | 14-17† | 14-17† |
| 13 | 4-5 | 9-9† | 9-9† | 16-17‡ | 16-17‡ | 16-17‡ |
| 14 | — | 4-5-13 | 4-9 | — | 16-17-18 | 17-18 |
| 15 | 5-5 | 5-5 | 5-9 | 15-17 | 15-16 | 15-16 |

*Recipient (R), retrieved allograft (A), and donor (D) genotypes at five short tandem repeats (CD4, VWA, FES, TPOX, and p53). Only short tandem repeat allelic patterns differing between donor and recipient are informative to identify the origin of retrieved allograft cartilage cells. Allele nomenclature refers to the number of repeats[27]. Cases 1 through 10: A = R, complete matching between retrieved allograft and recipient DNA; Cases 11 through 13: A = D, complete matching between retrieved allograft and donor DNA; and Cases 14 and 15: A = mixed, retrieved allograft has a mixed (donor and recipient matched) DNA profile. †The retrieved allograft DNA matches with recipient or donor DNA. ‡Short tandem repeat identical allele patterns in donor and recipient constitutional DNA are non-informative for typing analysis, having identity determination purposes.

**TABLE E-2 (*continued*)**

| Short Tandem Repeats | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FES | | | TPOX | | | p53 | | |
| R | A† | D | R | A† | D | R | A† | D |
| 11-11‡ | 11-11‡ | 11-11‡ | 8-11† | 8-11† | 8-8 | 8-9† | 8-9† | 7-9 |
| 10-13† | 10-13† | — | 11-11† | 11-11† | — | 8-8† | 8-8† | — |
| 10-12† | 10-12† | 12-12 | 11-11† | 11-11† | 8-8 | 8-8† | 8-8† | 8-9 |
| 10-13† | 10-13† | — | 8-8† | 8-8† | — | 7-9† | 7-9† | — |
| 10-12† | 10-12† | 10-11 | 8-11† | 8-11† | 8-9 | 8-8† | 8-8† | — |
| 10-11† | 10-11† | 12-12 | 8-8‡ | 8-8‡ | 8-8‡ | 8-8‡ | 8-8‡ | 8-8‡ |
| 11-11‡ | 11-11‡ | 11-11‡ | 9-11† | 9-11† | 8-8 | 7-9† | 7-9† | 7-8 |
| 10-12† | 10-12† | 11-11 | 8-11† | 8-11† | 9-11 | 8-8† | 8-8† | 7-7 |
| 11-13† | 11-13† | 11-11 | 8-8‡ | 8-8‡ | 8-8‡ | 9-9† | 9-9† | 7-8 |
| 12-12† | 12-12† | 10-11 | 9-11† | 9-11† | 8-9 | 7-8† | 7-8† | 8-9 |
| 10-12 | 11-13† | —† | 8-11‡ | 8-11‡ | —‡ | 8-10 | 8-8† | —† |
| 11-12‡ | 11-12‡ | 11-12‡ | 8-8‡ | 8-8‡ | 8-8‡ | 7-8 | 8-9† | 8-9† |
| 11-12 | 10-11† | 10-11† | 8-8‡ | 8-8‡ | 8-8‡ | 8-9 | 8-8† | 8-8† |
| — | 10-11 | 10-11 | — | 8-9 | 8-9 | — | 8-9 | 8-9 |
| 11-12 | 12-12 | 12-12 | 9-11 | 9-11 | 8-8 | 8-8‡ | 8-8‡ | 8-8‡ |

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**TABLE E-3 Relative mRNA Expression (Copy Number/100,000 GAPDH Copies) of Different Markers in Cartilage Allograft Samples and in Controls**

| | Markers* | | | | | | |
|---|---|---|---|---|---|---|---|
| Expression | Col II | Col IX | Col X | Aggr | SOX9 | Cath B | MMP-13 |
| Samples† | | | | | | | |
| 3 | 0 | 42 | 580 | 15,177 | 1992 | 81,225 | 13,679 |
| 8 | 333 | 8 | 40 | 16,724 | 3956 | 73,204 | 404 |
| 10 | 0 | 202 | 0 | 4 | 534 | 6121 | 0 |
| 15 | 57,038 | 34 | 10,224 | 38,157 | 1563 | 28,917 | 326 |
| 16 | 290,795 | 172 | 2435 | 20,448 | 1399 | 130,134 | 53,219 |
| 17 | 820 | 0 | 0 | 21,613 | 3983 | 80,107 | 3125 |
| Mean | 58,164 | 76 | 2213 | 18,687 | 2237 | 66,618 | 11,792 |
| CI† | 167-159,657 | 15-146 | 27-5731 | 9200-28,276 | 1192-3330 | 33,215-99,381 | 299-30,354 |
| Controls§ | | | | | | | |
| C1 | 72,262 | 592 | 54,337 | 408,405 | 64,618 | 61,132 | 0 |
| C2 | 469,134 | 0 | 29,730 | 265,3823 | 288,786 | 162,451 | 0 |
| C3 | 194,531 | 211 | 83 | 125,701 | 41,754 | 30,993 | 14 |
| C4 | 190 | 4 | 9 | 1722 | 910 | 40,895 | 1663 |
| C5 | 0 | 0 | 0 | 3280 | 169 | 83,509 | 1 |
| C6 | 1 | 4 | 0 | 258 | 76 | 100,696 | 5 |
| C7 | 10 | 0 | 101 | 18,428 | 11,502 | 402,782 | 1176 |
| C8 | 0 | 0 | 0 | 130,134 | 9606 | 365,533 | 52 |

*Col II = collagen type II, Col IX = collagen type IX, Col X = collagen type X, Aggr = aggrecan, SOX9 = sex-determining region Y-box 9, Cath B = cathepsin B, Col I = collagen type I, AP = alkaline phosphatase, RUNX2 = runt-related transcription factor 2, OC = osteocalcin, OP = osteopontin, and BSP = bone sialoprotein. †These are the retrieved allograft samples from Cases 3, 8, 10, 15, 16, and 17. ‡CI is the 95% bootstrap confidence intervals. §These are the controls of normal cartilage (C1 and C2), freshly isolated chondrocytes from normal cartilage (C3), cultured chondrocytes at P1 (C4) and P10 (C5), cultured synovial fibroblasts at P3 (C6), freshly isolated osteoblasts (C7), and bone marrow-derived mesenchymal stem cells (C8).

**TABLE E-3 (*continued*)**

| | | | Markers* | | | |
|---|---|---|---|---|---|---|
| Col I | AP | RUNX2 | OC | OP | BSP | |
| 1,492,853 | 22,531 | 34,628 | 271,321 | 1,308,643 | 419,887 | |
| 10,689,125 | 787 | 4544 | 347 | 855 | 0 | |
| 4124 | 0 | 0 | 0 | 31,864 | 1234 | |
| 4737 | 34 | 694 | 1160 | 75,262 | 5712 | |
| 1,837,917 | 9740 | 13,584 | 260,268 | 1,238,052 | 267,586 | |
| 5,571,524 | 4181 | 6983 | 837 | 2592 | 3540 | |
| 3,266,713 | 6212 | 10,072 | 88,989 | 442,878 | 116,326 | |
| 557,877-6,927,247 | 410-14,003 | 1977-21,846 | 395-20,1978 | 14,641-982,592 | 1739-278,268 | |
| 0 | 0 | 1184 | 592 | 85,264 | 663,456 | |
| 0 | 0 | 0 | 0 | 246,229 | 2,170,567 | |
| 168 | 0 | 59 | 226 | 9807 | 22 | |
| 13,870 | 25 | 17 | 49 | 4836 | 298 | |
| 46,652 | 9 | 99 | 144 | 13 | 176 | |
| 92,659 | 0 | 0 | 0 | 0 | 0 | |
| 431,691 | 165 | 4575 | 2916 | 1370 | 2665 | |
| 1,622,335 | 2418 | 14,063 | 505 | 28 | 4067 | |

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**TABLE E-4 List of ICRS-I Subscores for the Seventeen Analyzed Cases***

| | Subscores† | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surface | | | Matrix Staining | | | Cell Distribution | | |
| Cases | R | A | D | R | A | D | R | A | D |
| 1 | 0 | 2 | 3 | 2 | 2 | 3 | 0 | 1 | 2 |
| 2 | — | — | — | — | — | — | — | — | — |
| 3 | 0 | 0 | — | 3 | 2 | — | 0 | 1 | — |
| 4 | — | 3 | — | — | 1 | — | — | 1 | — |
| 5 | 0 | 3 | 3 | 1 | 2 | 2 | 1 | 0 | 2.5 |
| 6 | 0 | 3 | 3 | 1 | 2 | 2.5 | 0 | 3 | 2 |
| 7 | — | 3 | — | — | 1 | — | — | 1 | — |
| 8 | 0 | 2 | 3 | 1 | 2 | 2 | 0 | 1 | 2 |
| 9 | — | 3 | — | — | 2 | — | — | 3 | — |
| 10 | — | 0 | 3 | — | 2 | 2 | — | 0 | 2 |
| 11 | — | 3 | — | — | 3 | — | — | 3 | — |
| 12 | 0 | 2 | 3 | 3 | 2 | 2 | 1 | 1 | 2 |
| 13 | 0 | 3 | 3 | 1 | 2 | 2 | 0 | 3 | 2.5 |
| 14 | — | 3 | 3 | — | 3 | 2 | — | 3 | 2 |
| 15 | 0 | 3 | 3 | 2 | 2 | 2.5 | 1 | 3 | 2 |
| 16 | 0 | 0 | 3 | 2 | 2 | 2 | 1 | 2 | 2 |
| 17 | — | 0 | — | — | 2 | — | — | 0 | — |

*R = recipient, A = retrieved allograft, and D = donor. †The range of subscores is 0 to 3 points. ‡The range of the total score is 0 to 18 points.

**TABLE E-4 (*continued*)**

| | | | Subscores† | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell Viability | | | Subchondral Bone | | | Cartilage Mineralization | | | Total Score† | | |
| R | A | D | R | A | D | R | A | D | R | A | D |
| 1 | 1 | 3 | 3 | 2 | 3 | 0 | 0 | 3 | 6 | 8 | 17 |
| — | — | — | — | — | — | — | — | — | — | — | — |
| 1 | 3 | — | 2 | 2 | — | 0 | 0 | — | 6 | 8 | — |
| — | 1 | — | — | 3 | — | — | 0 | — | — | 9 | — |
| 1 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 3 | 6 | 11 | 16.5 |
| 1 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 3 | 5 | 14 | 16.5 |
| — | 1 | — | — | 3 | — | — | 0 | — | — | 9 | — |
| 0 | 1 | 3 | 2 | 3 | 3 | 0 | 0 | 3 | 3 | 9 | 16 |
| — | 3 | — | — | 3 | — | — | 0 | — | — | 14 | — |
| — | 1 | 3 | — | 2 | 3 | — | 0 | 3 | — | 5 | 16 |
| — | 3 | — | — | 3 | — | — | 0 | — | — | 15 | — |
| 1 | 1 | 3 | 3 | 2 | 2 | 0 | 0 | 3 | 8 | 8 | 15 |
| 1 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 3 | 5 | 14 | 16.5 |
| — | 3 | 3 | — | 3 | 3 | — | 0 | 3 | — | 15 | 16 |
| 1 | 3 | 3 | 2 | 2 | 3 | 0 | 3 | 3 | 6 | 16 | 16.5 |
| 1 | 1 | 3 | 2 | 3 | 3 | 0 | 0 | 3 | 6 | 8 | 16 |
| — | 3 | — | — | 2 | — | — | 0 | — | — | 7 | — |

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## Appendix

### *Surgical Technique*

Surgical treatment was performed in two steps, one for the graft preparation and one for surgery in the recipient. On a separate table, the harvested ankle had all soft tissues removed. Care was taken not to damage the cartilage surface. The fibula was removed and the medial malleolar surface was cut by means of a specifically designed jig held in place by a Kirschner wire. Then the tibial surface was first prepared by using two 2-mm Kirschner wires to define the planes of the cut and a standard pneumatic saw. The cut was performed at a proper level to obtain a 1-cm-thick osteochondral surface. The talar surface was then prepared with the same method, taking care to obtain a 1-cm-thick talar dome surface.

The patient was then placed supine on the operating table with a tourniquet at the proximal thigh. The ankle joint was approached through an anterior midline incision between the extensor hallucis longus and tibialis anterior tendons. Osteophytes and fibrous tissues were removed. The medial malleolar surface was prepared with use of the same specifically designed jig that had been previously used for the cut of the donor surfaces. Then two 2-mm Kirschner wires were positioned and were checked by fluoroscopy, both on the tibia and the talus, at the same distance from the joint line used in preparing the graft, and both the distal tibial and talar surfaces were cut and were removed. Allograft surfaces were positioned in the host ankle and were fixed under fluoroscopic control with two twist-off screws (DePuy, Warsaw, Indiana) on each side. The graft was tested for stability and range of motion. A standard closure was performed and postoperative radiographs were made.

### *Genotyping*

The technique involves DNA amplification at five short tandem repeats (microsatellites), polymorphic DNA loci present throughout the genome and used for human identification. Because of the high short tandem repeat polymorphism in the population, by analyzing a combination of short tandem repeats, there is an extremely high chance (near 100%) to obtain a pattern of alleles specific for one individual, thus allowing discrimination between recipient and donor DNA and identification to which of these the allograft matches. Genotyping includes PCR amplification of the short tandem repeat loci and allele identification by electrophoresis. Retrieved allograft genotype determination was obtained by a comparison of allograft allele patterns to recipient and donor constitutional DNA allele patterns at each short tandem repeat.

### *Likelihood Ratio*

The likelihood ratio test is a statistical test used to compare the fit of two hypotheses, one of which (the null hypothesis) is a special case of the other (the alternative hypothesis). The test is based on the likelihood ratio, which expresses how many times more likely the data are under one hypothesis than the other. Here we applied this test for identity purposes in cases in which donor DNA was not available and we wanted to determine if the retrieved allograft did or did not match with the recipient. We therefore used, as alternative hypotheses, the hypothesis of allograft matching with the recipient compared with the hypothesis that the allograft was from an unknown person of the population (in this case, the missing donor). The likelihood ratio was calculated as 1/p, where p represents the population frequency of the short tandem repeat profile (that is the product of the genotype frequency of each of the five analyzed short tandem repeats, based on published Caucasian population allele frequencies). The likelihood ratio obtained was then compared with a critical value that is typically 1000, indicating that the hypothesis of identity is 1000 times more likely than that of no identity. Because the likelihood ratios that we calculated were far greater than 1000 ($8.8 \times 10^5$ for Case 2 and $3.9 \times 10^7$ for Case 4, which means that it is $8.8 \times 10^5$ and $3.9 \times 10^7$ more likely that DNA belongs to the recipient than to some unknown person from the population), we accepted the hypothesis of the identity of the allograft with the recipient.

### *Cluster Analysis*

Cluster analysis is the task of grouping a set of objects in such a way that objects in the same group (called a cluster) are more similar to each other than to those in different groups. It can be achieved by various algorithms that differ in their notion of what constitutes a cluster and how to efficiently find them. Hierarchical clustering is based on the core idea of objects being more related to nearby objects than to objects farther away. It seeks to build clusters using a measure of dissimilarity between sets of observations, by means of algorithms connecting objects to form clusters based on their distance. In our case, cosine similarity as a matrix of distances was used. Hierarchical cluster analysis is a common technique for statistical data analysis. At different distances, different clusters will form, which can be represented with use of a dendrogram providing an extensive hierarchy of clusters that merge with each other at certain distances. ■