

PROCHONDRIX® FRESH
OSTEOCHONDRAL ALLOGRAFT
MAINTAINS VIABLE CHONDROCYTES,
OSTEOBLASTS AND A MINERALIZED
MATRIX NECESSARY TO SUPPORT
BONE AND CARTILAGE FORMATION

Anna-Laura Nelson, MS; Carolyn Barrett, BS, MBA;
Ramasamy Sakthivel, PhD, MBA
AlloSource®, Centennial, CO

ProChondrix® Fresh Osteochondral Allograft Maintains Viable Chondrocytes, Osteoblasts and a Mineralized Matrix Necessary to Support Bone and Cartilage Formation

Anna-Laura Nelson, MS; Carolyn Barrett, BS, MBA; Ramasamy Sakthivel, PhD, MBA
AlloSource®, Centennial, CO

Introduction

Living osteochondral allografts not only supply a matrix conducive for recipient cells to support regeneration of cartilage, but they also provide the cells necessary to facilitate remodeling of the subchondral bone, including viable chondrocytes and osteoblasts. Current practices of osteochondral allograft implantations often require the removal of recipient subchondral bone; however, for some patients where the subchondral bone is still healthy, a thinner osteochondral allograft may be utilized in order to avoid the unnecessary removal of subchondral bone and allow bone to bone healing. ProChondrix®, a living cellular allograft, is indicated to aid in the repair of surface articular cartilage lesions. It was therefore necessary to examine for the presence of osteoblast progenitor cells to determine ProChondrix's abilities of reintegrating into the subchondral bone region and thus, its capabilities of serving as a thin osteochondral allograft.

Methods

All fresh osteochondral allografts (ProChondrix®, AlloSource®, Centennial, CO) were recovered from human donors consented for research and prepared at various diameters, ranging from 7 to 20 mm, all with 1 mm thickness and laser etched with a 1.5 mm square pattern. Fresh tissue was used for all studies except flow cytometry, where ProChondrix samples were tested up to one week after the 35 day shelf life expiration. All samples were recovered in Chondrocyte Growth Medium (Cell Applications, San Diego, CA). Non-viable ProChondrix controls were prepared by storing expired grafts in 70 percent isopropyl alcohol (IPA) for at least 12 hours.

Alkaline Phosphatase stain (AP, Vector Laboratories, Burlingame, CA) was applied to the entire cartilage graft, while Von Kossa stain (VK, IHC World, Ellicott City, MD) was applied to 5 µm thick sections and placed under UV light for 1.5 hours. Flow cytometry was used to quantify osteoblast progenitor cells (anti-osteocalcin, BD BioSciences, San Jose, CA). Antibodies were allowed to react for one hour prior to two washes and quantification. ProChondrix grafts were embedded in a tissue freezing compound, sectioned and stained with antibodies to Osteopontin (GeneTex, Irvine, CA), a marker for osteoblasts, and Collagen II (Proteintech, Chicago, IL), a marker for cartilage. Slides were fixed in cold 50 percent acetone, 50 percent methanol solution for five minutes, washed with Phosphate Buffered Saline (PBS) and stained with primary antibodies at a concentration of 1:200, and incubated overnight at 40 degrees Celsius. The slides were washed with PBS, incubated with secondary antibodies, fluorescein isothiocyanate (FITC, Invitrogen, Waltham, MA) and tetramethylrhodamine isothiocyanate (TRITC, Abcam, Cambridge, UK), at a concentration of 1:100 for two hours. Slides were washed and mounted using 4',6-diamidino-2-phenylindole (DAPI) coated cover slips. Slides were imaged using confocal and epifluorescence microscopy.

Results

OSTEOGENIC ACTIVITY AS DEMONSTRATED BY AP STAINING

Alkaline Phosphatase is an enzyme involved in osteogenesis and plays an early role in the process of calcification.¹ ProChondrix® allografts showed dense AP staining, giving further insight into the osteogenic activity within each sample tested (Fig. 1).

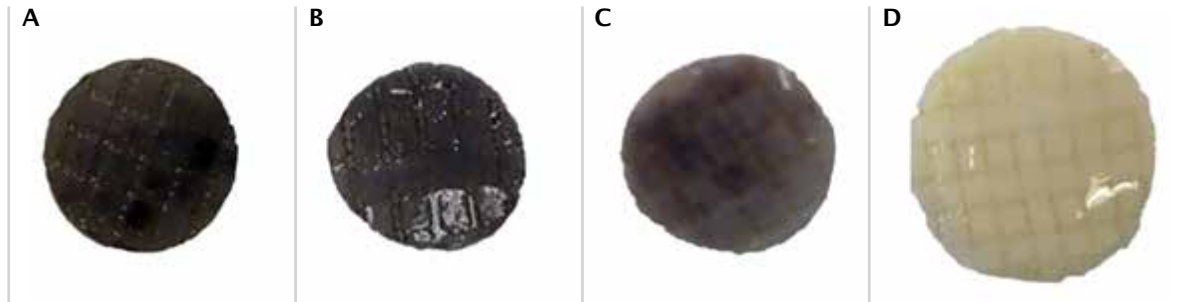


Figure 1. A, B, C. ProChondrix allografts stained with Alkaline Phosphatase on three different donors, demonstrating the presence of osteogenic activity. D. Non-viable allograft stained with AP, serving as a control, displaying the variation in osteogenic activity between viable and non-viable ProChondrix grafts.

MINERALIZATION OF PROCHONDRIX ALLOGRAFTS AS DEMONSTRATED BY STAINING WITH VON KOSSA STAIN

Calcium deposition is a characteristic of bone matrix formation and a signifier of osteoblast differentiation. VK staining measures the extent of mineral deposition within the matrix, an indirect measurement of calcium content. **Figure 2** depicts the VK stain for ProChondrix allografts, indicating mineral deposition as shown by dark brown/black staining at the chondral bone junction.

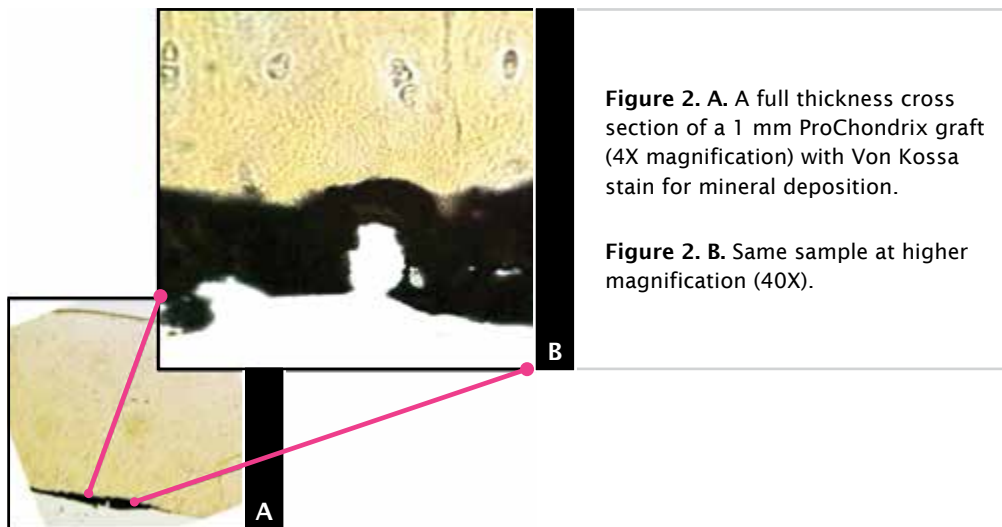


Figure 2. A. A full thickness cross section of a 1 mm ProChondrix graft (4X magnification) with Von Kossa stain for mineral deposition.

Figure 2. B. Same sample at higher magnification (40X).

QUANTITATIVE ANALYSIS OF OSTEOBLASTS IN PROCHONDRIX® BY FLOW CYTOMETRY

The presence of specific markers for osteoblast progenitor cells was qualitatively and quantitatively measured using confocal microscopy and flow cytometry, respectively. Osteocalcin is a protein specifically secreted by osteoblasts, and serves as a marker for osteogenic activity.² All ProChondrix allograft samples were tested up to one week after the 35-day shelf life, representing a graft with less cellular activity as compared to standard production grafts. Osteocalcin expression was detected in all except for one ProChondrix graft as shown in **Table 1**.

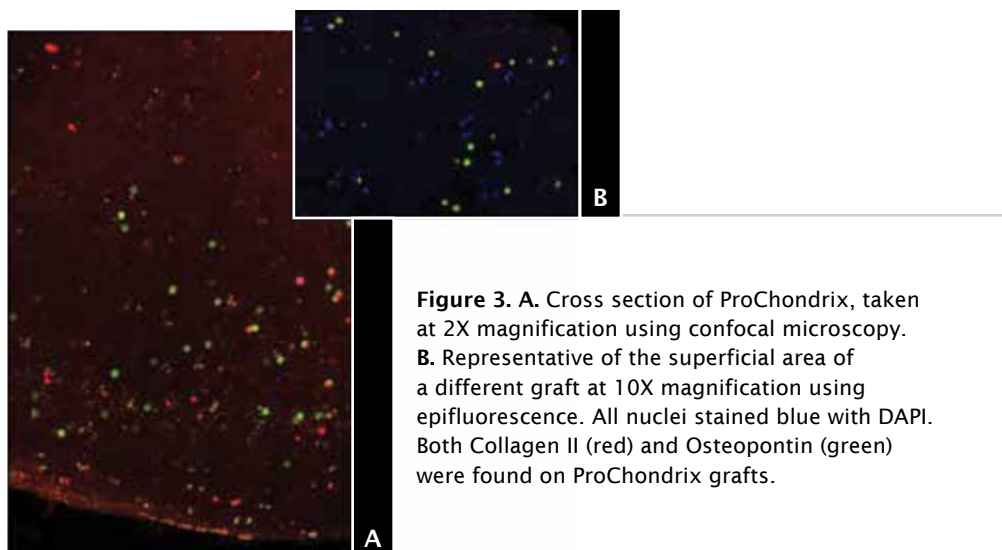
| Osteocalcin expression levels | | |
|---------------------------------|--------------------|------------------------|
| | Viable ProChondrix | Non-viable ProChondrix |
| Number Samples | 8 | 3 |
| Average OCN Cell Count | 28,147 | 161 |
| Standard Deviation | 35,416 | 845 |
| Average OCN Per mm ³ | 22.4 | 0.13 |

Table 1. Flow cytometry results for eight ProChondrix samples. Osteocalcin (OCN) signifies osteoblast activity.

Donor-to-donor variation contributed to both the variation seen in Osteocalcin expression, as well as the level of digestion between grafts. This variation in the level of digestion may account for both the high standard deviation, as well as contributing to the lack of Osteocalcin expression on one of the grafts.

QUALITATIVE ANALYSIS OF OSTEOBLASTS IN PROCHONDRIX BY MICROSCOPY

To compare the osteogenic and chondrogenic activity, ProChondrix grafts were stained with Osteopontin to test for the presence of osteoblast progenitor cells, and Collagen II for the presence of chondrocytes. Similarly to Osteocalcin (OCN) expression, Osteopontin expression indicates the presence of osteoblasts and osteoblast differentiation.³ **Figure 3** depicts immunohistochemical staining of ProChondrix's osteogenic (green) and chondrogenic activity (red).



Discussion

Osteochondral allografts provide a matrix consisting of both bone and cartilage, as well as viable chondrocytes and osteoblasts necessary to facilitate regeneration of both the subchondral bone and the adjacent cartilage. To determine the level of osteogenic activity within ProChondrix®, various methods were utilized to test for osteogenic activity and bone mineralization within the matrix. Additionally, osteoblast progenitor expression levels were identified and quantified using confocal microscopy and flow cytometry. The data suggests ProChondrix grafts not only display osteogenic activity beyond the 35-day shelf life, but also maintain viable osteoblast progenitor cells (**Fig. 1, 2 and 3**). ProChondrix was also shown to display calcium deposition within its matrix, further signifying ProChondrix is conducive to osteoblast progenitor growth and differentiation (**Fig. 2, Table 1**). This data allows us to speculate that ProChondrix (AlloSource®, Centennial, CO) may serve as an acceptable adult osteochondral allograft for repair of superficial chondral defects.

References

1. Golub E, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. *Curr Opin Orthop.* 2007;18:444-448.
2. Cantatore A, Corrado A, Grano M, Quarta L, Colucci S, Melillo N. Osteocalcin synthesis by human osteoblasts from normal and osteoarthritic bone after vitamin D3 stimulation. *Clin Rheumatol.* 2004 Apr;23(6):490-495.
3. Weinreb M, Shinar D, Rodan G. Different pattern of alkaline phosphatase, osteopontin and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J Bone and Miner Res.* 1990 Aug;5(8): 831-842.



6278 S Troy Cir
Centennial, CO 80111

MAIN 720. 873. 0213
TOLL FREE 800. 557. 3587
FAX 720. 873. 0212

allosource.org
prochondrix.org