

EXTRACELLULAR MATRIX AND GROWTH FACTORS EXPRESSION IN PROCHONDRIX® IS COMPARABLE TO UNPROCESSED ADULT CARTILAGE: A RATIONALE FOR CONSIDERING SIGNALING DYNAMICS

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Extracellular Matrix and Growth Factors Expression in Prochondrix® is Comparable to Unprocessed Adult Cartilage: A Rationale For Considering Signaling Dynamics

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Introduction

Articular cartilage is a unique tissue type consisting of a dense matrix of collagen and proteoglycans that serves to cushion and lubricate the joint. The complex signaling interaction of extracellular proteins, such as growth factors and cytokines, along with chondrocytes and the extracellular matrix (ECM), allows them to cooperatively maintain the tissue's health, structure and function. Articular cartilage lesions, particularly those affecting the knee, secondary to acute trauma or osteoarthritis, remain a significant clinical challenge due to the poor intrinsic repair capacity of this highly specialized tissue. ProChondrix® is a cellular 3-dimensional fresh cartilage matrix made from adult donors. It is laser perforated to allow for outgrowth of the viable chondrocytes that aid in cartilage regeneration and reliably restore the natural articular cartilage integrity. Both adult and juvenile cartilage allografts have been utilized in adult cartilage replacement procedures, however, the signaling environments of these different tissues have not been examined in-depth. The purpose of this study was to characterize the different levels of chondrocyte outgrowth and signaling factors in both adult (fresh unprocessed and fresh processed (ProChondrix)) and juvenile cartilage tissues.

Methods

CHARACTERIZATION OF ECM AND CHONDROCYTE OUTGROWTH FROM PROCHONDRIX EXPLANTS

To characterize and compare chondrocyte outgrowth and matrix protein production between adult ProChondrix and juvenile donors, a 12-week explant study was performed. Three research-consented adult donors (15-35 y/o) and two research-consented juvenile donors (1 mo-12 y/o) were obtained. Samples were sliced by hand into 1 mm thick sheets and laser cut into 6 x 6 mm sheets with 2 mm squares (5 samples per donor) and glued to a 12-well plate using TISSEL (Baxter, Deerfield, IL); Chondrocyte media (Cell Applications, San Diego, CA) was then added and changed twice weekly. A 1:10 ratio of Presto Blue (Invitrogen, Carlsbad, CA) to media was used for weekly cell counting. Collagen II immunohistochemistry was performed on samples after the 12-week time point, as well as sGAG assay (Kamiya Biomedical Company, Seattle, WA), Hydroxyproline assay (BioVison, Milpitas, CA) and DNA analysis with a Pico Green Assay (Invitrogen, Grand Island, NY). Hydroxyproline assay was used to determine the content of collagen in the explants. As roughly 13 percent of cartilage is hydroxyproline, the content was divided by 0.13 to obtain the collagen content.¹ DNA content was calculated to estimate the total number of cells, based on the assumption that there is approximately 6 pg DNA per cell.

CHARACTERIZATION OF GROWTH FACTOR EXPRESSION IN CARTILAGE TISSUE HOMOGENATES

ProChondrix grafts that had undergone final processing and were at their maximum shelf life, as well as unprocessed fresh adult and unprocessed fresh juvenile cartilage, were tested for growth factor expression. Ten research consented donors from each sample group were cut into approximately 1 mm square cubes and placed in a volume of RayBiotech Lysis buffer with Thermo Scientific Halt Protease Inhibitor cocktail based on the tissue weight. A VWR VDI 25 Homogenizer was utilized to grind the tissue. The homogenate was kept on ice for 2 hours to allow for protein extraction, then sonicated for 5 minutes and passed through a cell strainer to remove debris. The homogenates were then centrifuged at 12000 xg for 10 minutes and the supernatant collected, aliquoted and stored at -80°C until use.

The presence of growth factors, such as IGF-1, bFGF, TGF- β , BMP-2, PRG4 (Superficial Zone Protein) and BMP-7 were analyzed in tissue homogenates by ELISA using RayBiotech, Novex, R&D Systems, Enzo Lifesciences and Cloud Clone Corp ELISA sandwich kits. The procedure recommended for each kit was followed. All washes were performed with a Biotek 405 Select TS plate washer. Colorimetric readings were gathered immediately at 450 nm with a Biotek Synergy H1 Hybrid Reader and normalized. Curve fitting was performed with built in software on the reader.

STATISTICAL ANALYSIS

Two sample t-tests assuming unequal variance were performed between the fresh cartilage group and the other cartilage groups to determine if the distributions had the same mean. The null hypothesis being that the two groups had the same mean.

Results

CHARACTERIZATION OF ECM AND CHONDROCYTES OUTGROWTH FROM PROCHONDRIX EXPLANTS

The 12-week explant study (**Fig. 1**) showed the juvenile cartilage sheets started with more cells and grew significantly faster than the ProChondrix sheets between the Day 1 and Week 6 time points. After 6 weeks the juvenile sheet growth rate leveled off. The ProChondrix chondrocytes grew at a steady pace reaching the same number as the juvenile cells at Week 9, continuing through Week 12. The hydroxyproline and sGAG assays (**Fig. 2** and **3**) showed both groups exhibited similar capabilities of collagen and GAG production. Collagen II IHC staining (**Fig. 4**) and alcian blue histology staining (**Fig. 5**) confirmed both groups' chondrocytes produced collagen II and GAGs, however the juvenile collagen produced appears to be disorganized and immature compared to the adult. The alcian blue staining shows that while both groups of chondrocytes produce GAGs, there is a clear difference in the morphology between the adult and juvenile cells, with the juvenile cells appearing more spindle-like than the ProChondrix chondrocytes.

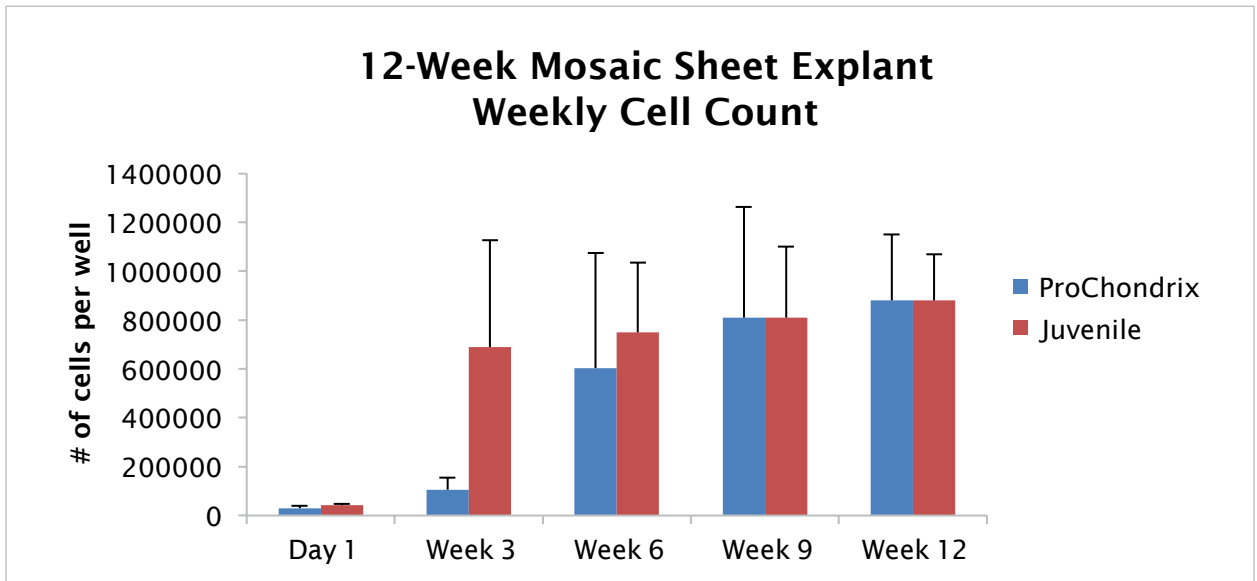


Figure 1. Weekly cell counts of explanted samples.

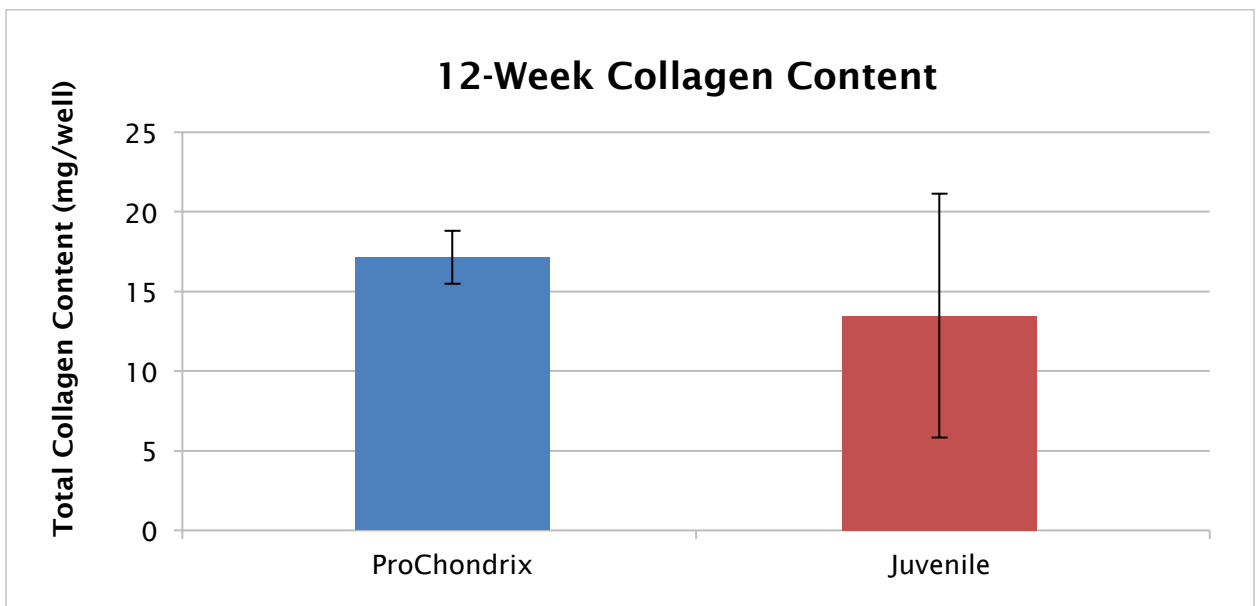


Figure 2. A comparison of collagen content at 12 weeks.

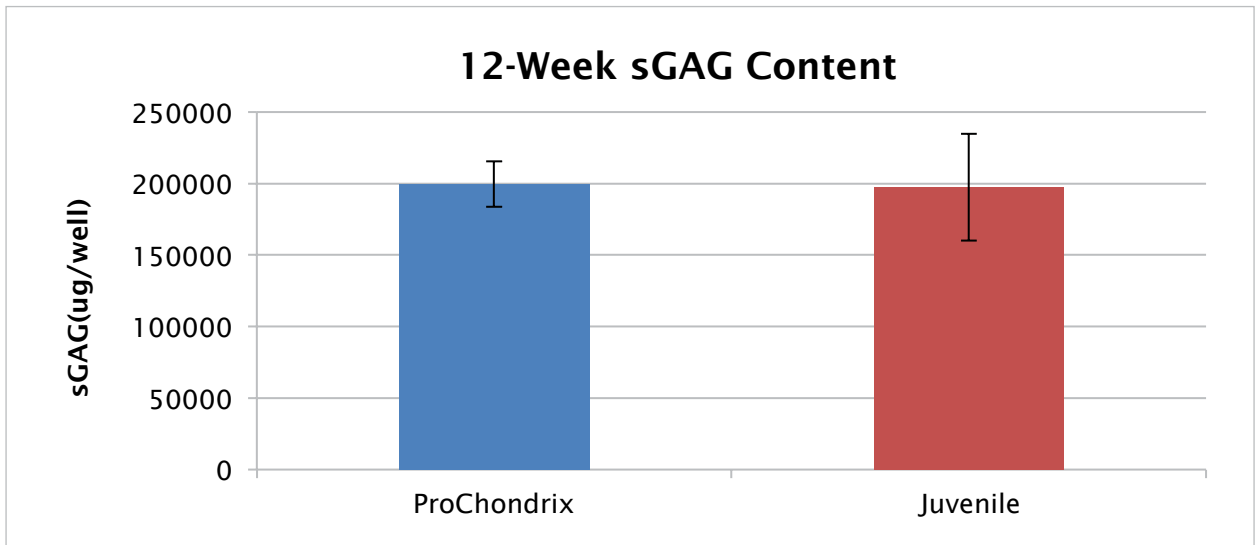


Figure 3. A comparison of sGAG content at 12 weeks

ProChondrix 12-Week Collagen II ProChondrix 12-Week Collagen II

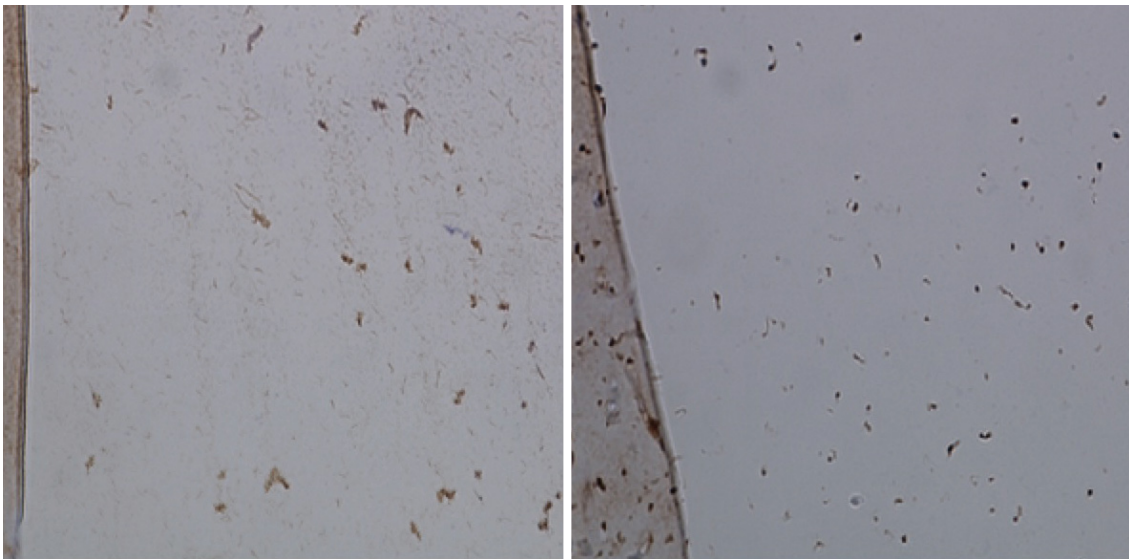


Figure 4. A comparison of collagen II using IHC staining in the cellular outgrowth of ProChondrix and juvenile grafts at 12 weeks.

ProChondrix 12-Week GAG

Juvenile 12-Week GAG

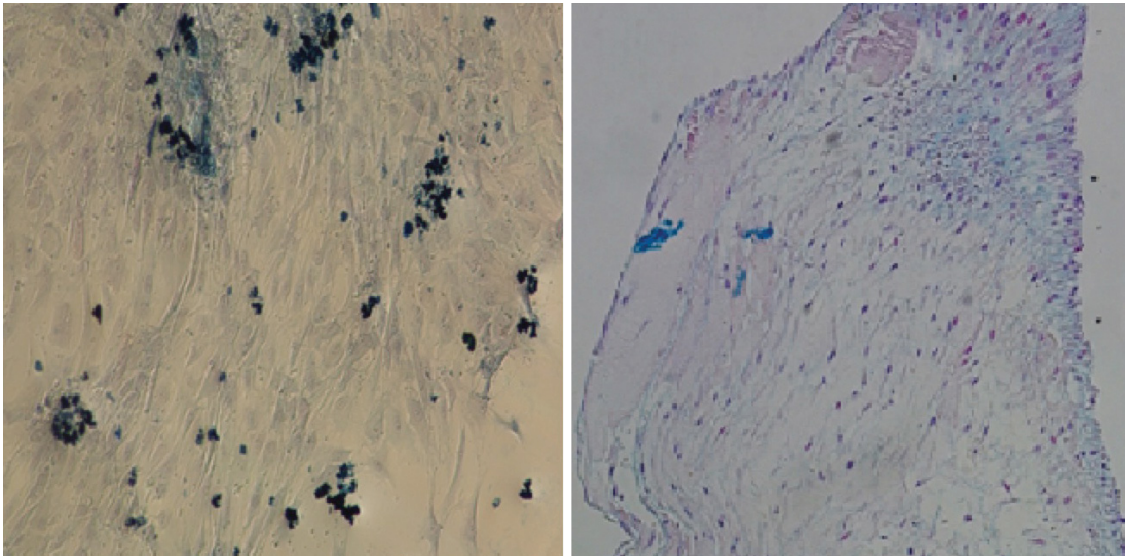


Figure 5. A comparison of Alcian blue staining showing GAG content at 12 weeks in ProChondrix and juvenile samples (10x).

GROWTH FACTOR EXPRESSION IN CARTILAGE TISSUES

The averaged results of the concentrations from the assays are displayed in **Table 1**. Juvenile cartilage consistently displayed higher levels of proteins in several different areas including IGF-1, bFGF, TGF- β and PRG4, as compared to both the processed cartilage of ProChondrix and unprocessed cartilage of the adult. In the case of IGF-1, the levels are almost an order of magnitude higher than the other groups. ProChondrix displayed higher levels of BMP-7 while the other cartilage groups expressed virtually no BMP-7. BMP-2 was the highest in fresh unprocessed adult followed by ProChondrix. The comparisons between the expression levels of the different factors are shown in **Figure 6**.

Table 1. Growth factor concentrations in different cartilage compositions.

Growth Factor	ProChondrix® at 35-day shelf life	Fresh Unprocessed Adult	Juvenile
IGF-1 (pg/g tissue)	300	400	3,700
bFGF (pg/g tissue)	30,100	21,700	45,000
TGF- β (pg/g tissue)	4,400	5,700	9,000
BMP-2 (pg/g tissue)	1,600	4,700	1,150
PRG4 (pg/g tissue) (Superficial Zone Protein)	8,600	8,800	10,800
BMP-7 (pg/g tissue)	40	10	20

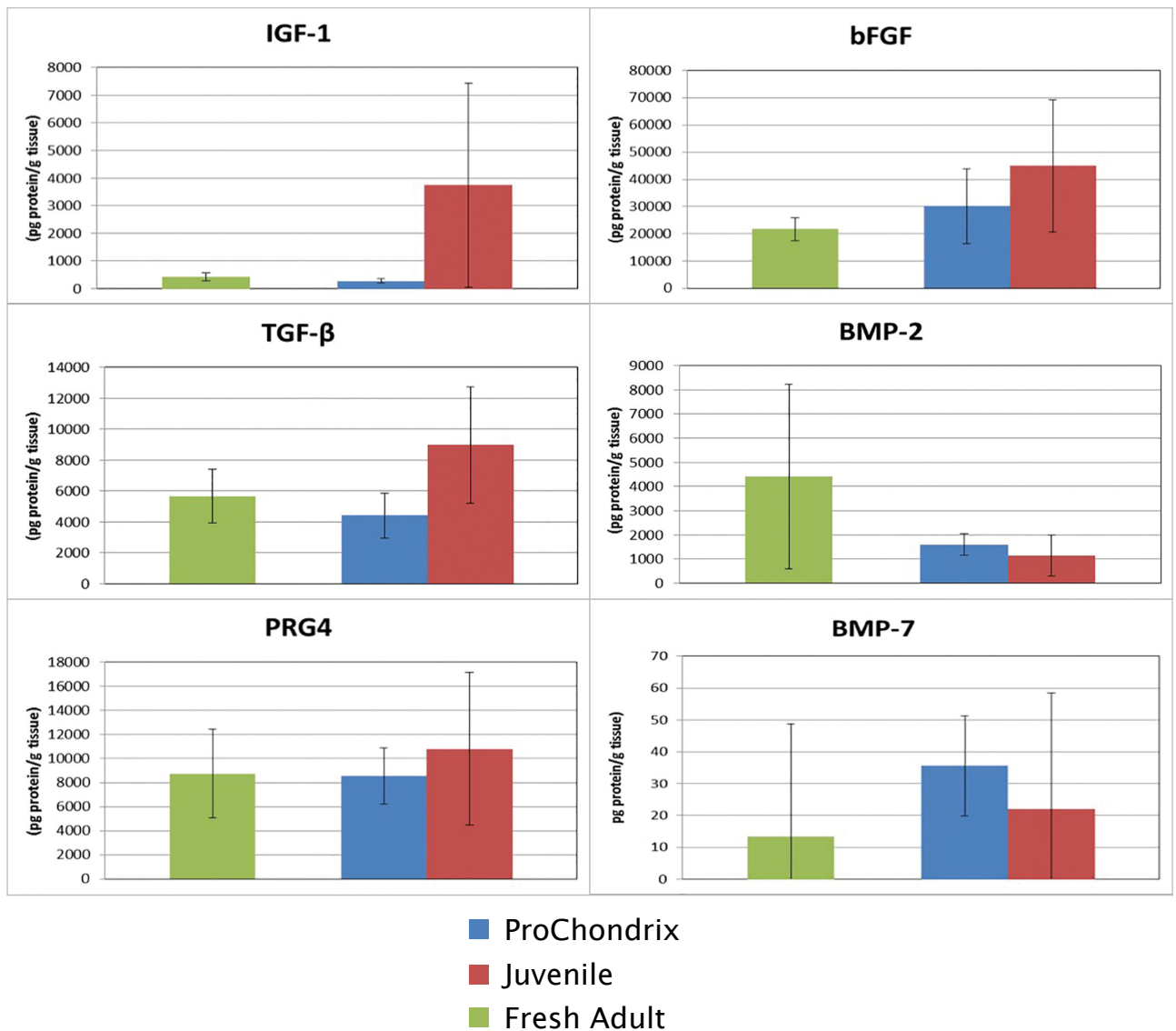


Figure 6. Bar plots of concentrations of different cartilage compositions.

When the populations of protein concentrations were compared with a two sample t-tests for unequal variance with an alpha of 0.05, several differences were revealed. Comparing juvenile to fresh unprocessed adult, only the population of PRG4 and BMP-7 were statistically similar. Comparing ProChondrix to fresh unprocessed adult, all values was statistically similar except IGF-1. The results of this analysis are summarized in **Table 2**.

Table 2. P-values for two sample t-test with unequal variance between fresh adult cartilage and the other cartilage groups.

	ProChondrix	Juvenile
bFGF	0.1047	0.00397
TGF-β	0.1534	0.000066
IGF-1	0.0408	0.007793
PRG4	0.5397	0.333298
BMP-7	0.0813	0.432787
BMP-2	0.0709	0.042601

Discussion

In this study, extracellular matrix and chondrocyte outgrowth potential of ProChondrix® Fresh Cartilage Allograft and juvenile cartilage sheets were compared. Adult cartilage is a largely quiescent tissue without a direct blood supply, where widely dispersed chondrocytes work relatively slowly to produce and breakdown the cartilage matrix.² Juvenile chondrocytes are more densely clustered, there is a limited blood supply in the tissue and there is less of a distinct transition point between cartilage and bone. These differences are designed to support the continued growth of bone and cartilage within the individual. Not only does this lead to different chondrocyte behavior between the adult and juvenile tissue,^{3,4} there are also distinct differences in the protein makeup of the cartilage and behavior of chondrocytes throughout all the stages of juvenile growth.⁵ While the signaling of juvenile chondrocytes is important in the generation of the developing osteochondral region of juveniles, it does not fully reflect the makeup of adult tissue. The selection of allograft for adult patients in which the growth factor levels more closely resembles adult cartilage may be an important consideration in order to complement the body's own natural healing mechanism. It is possible that transplanted juvenile cartilage allografts may promote excessive osteochondral growth in the adult recipient, leading to improper graft incorporation or other unexpected postoperative complications.

In examining the levels of growth factors in different sources of cartilage tissue, we have observed marked differences. IGF-1 was almost an order of magnitude greater in juvenile cartilage than for either unprocessed adult tissue or ProChondrix. High IGF-1 expression could be important to meet the need for the rapid anabolic growth in juvenile cartilage. This is beneficial in juveniles, however there is a large amount of research pointing to excessive IGF-1 being a causative factor in condylar hyperplasia in adults.⁶ High levels of TGF- β are related to growth in juvenile cartilage.⁷ We have also observed a similar trend in our data on TGF- β expression in juvenile cartilage. In adults there is a different signaling paradigm in which high levels of TGF- β have been shown to be related to osteoarthritis development^{8,9} and the inhibition of TGF- β expression in adult has been shown to reduce osteoarthritis symptoms.¹⁰ The lack of statistically similar levels of BMP-2 from juvenile cartilage to adult indicates another difference in the signaling needs required for juvenile versus adult cartilage. BMP-2 serves the purpose of lowering proliferation and driving chondrogenesis¹¹ and the lack of its signaling in juvenile cartilage may lead to hyperplasia in adults. This evidence provides a rationale for using a tissue graft in which signaling dynamics are similar to unprocessed adult tissue.

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