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Growth factor effects on passaged TMJ disk cells in monolayer and pellet cultures

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Structured Abstract

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Objectives – Previously, we demonstrated rapid changes in temporomandibular joint (TMJ) disk gene expression during monolayer expansion. This study's objective was to investigate the ability of pellet culture and growth factors to rescue TMJ disk gene expression changes.

Design – Temporomandibular joint disk cells were isolated from mature porcine tissue and passaged up to five times. At each passage, 300 000 cells were placed in a monolayer or pellet culture environment before being exposed to transforming growth factor-beta 3 (TGF-β3) (5 ng/ml), TGF-β1 (5 ng/ml), and insulin-like growth factor I (IGF-I) (10 ng/ml).

Outcome Measure – After 24 h, gene expression was analyzed via reverse transcriptase-polymerase chain reaction (RT-PCR).

Results – Pelleting was detrimental to TMJ disk gene expression, marked by gene expression decreases in collagen type I (5.5-fold), aggrecan (1.4-fold), decorin (0.73-fold), and biglycan (0.73-fold) relative to monolayer cultures. IGF-I, TGF-β1, and TGF-β3 demonstrated limited ability to rescue TMJ disk gene expression in the pellet culture. In monolayer, TGF-β3 and TGF-β1 increased decorin and biglycan gene expression relative to passaged controls. Collagen type I expression, the TMJ disk's primary matrix constituent, was highest in TGF-β3 cultures; however, differences were not statistically significant.

Conclusion – These results indicate that pellet cultures are a poor choice for TMJ disk tissue engineering, and the effects of TGF-β1, TGF-β3, and IGF-I on TMJ disk gene expression are minimal relative to passaging and pelleting effects.

Key words: cell culture technique; fibrocartilage; growth factors; temporomandibular joint disk; tissue engineering

Introduction

Temporomandibular joint (TMJ) dysfunction is a clinical syndrome associated with acute and/or chronic pain. Persons suffering from TMJ dysfunction may experience an inability to achieve functional mandibular movements, severe remodeling of articular tissues, and coinciding incidents of pain such as headaches (1). The pathogenesis of TMJ disorders is multifactorial and incompletely understood (2). Thus, treatment of TMJ disorders is controversial, and a variety of surgical treatments have been proposed and utilized (3), aiming to restore essential joint motions and reduce pain.

An important element in the debate of TMJ treatment modalities is the TMJ's central tissue, the TMJ disk. This fibrocartilaginous tissue likely aids in joint motions by lubricating the surfaces of motion, decreasing incongruence between the articulating surfaces, and dissipating loads and impact within the joint (4). However, when displaced, malformed, or perforated, this tissue may become obstructive, resulting in severe joint pain, crepitation, and loss of functional joint motions (1). Many TMJ treatment modalities focus on replacing, reshaping, and/or repositioning the TMJ disk to correct internal derangements of the joint (5–7). When the TMJ disk is beyond repair, the obstructive tissue must be removed. Post-discectomy, disk replacement may be attempted via an alternative tissue, such as autogenous fat, or the joint space may be left empty. Hereafter, joint remodeling is common.

Implantation of a viable TMJ disk equivalent may improve current treatment modalities by restoring the joint functions provided by a healthy TMJ disk (8). As synthetic TMJ disk implants have a dark history (9–11), tissue engineering may provide an avenue by which a biologically equivalent TMJ disk may be created (8). Tissue engineering aims at generating neotissue capable of restoring native structure–function relationships; this is commonly achieved by combining cells, biofactors, and a scaffolding material either *in vitro* or *in vivo*. Thus far, TMJ disk tissue engineering efforts have focused on scaffolds seeded with autologous, terminally differentiated cells *in vitro* (12). Within the knee joint, matrix-induced autologous chondrocyte implantation (MACI) has been used with some success (13). In this procedure, autologous chondrocytes are derived from a biopsy of the non-weight-bearing cartilage, isolated, and then expanded *in vitro*. With refinement, it may be possible to utilize a similar procedure in TMJ repair, thus allowing for the restructure or reshaping of degenerated cartilage surfaces.

The selection of a viable cell population is essential for the success of tissue engineering approaches, both *in vitro* and *in vivo*. Stem cells, both adult and embryonic, have a great potential to yield a large population of healthy cells; however, differentiating stem cells to an appropriate phenotype can be quite complex (14–16). Healthy terminally differentiated cells may be difficult to identify in the patient, and if available, these cells may require multiple expansions to yield appropriate cell numbers (17). Furthermore, expansion of cells *in vitro* may lead to dedifferentiation and loss of phenotype (18).

Changes in the expression levels of specific extracellular matrix (ECM) proteins during *in vitro* expansion have been noted in TMJ disk cells (19). Rapid losses in collagen type I, collagen type II, and aggrecan expression occurred with passage while decorin expression rapidly increased (19). Prior to these findings, autologous TMJ disk cells were suggested as a viable option for tissue engineering as the TMJ disk is populated primarily by fibroblast-like cells (70%) with the remainder mostly chondrocyte-like cells (8,12,20). Growth factors may serve as a viable avenue to rescue TMJ disk gene expression. Previously, growth factors have shown increased matrix deposition in fibrocartilage tissue engineering studies (21–24). These effects, however, can differ between monolayer and tissue engineering environments (21–24).

Motivated by the rapid changes in TMJ disk gene expression caused by passaging, in this study, we investigate the potential of appropriate growth factors to serve as a rescue vehicle for TMJ disk gene expression. In doing so, we describe the effects of transforming growth factor-beta 3 (TGF-β3), transforming growth factor-beta 1 (TGF-β1), and insulin-like growth factor I (IGF-I) on passaged TMJ disk gene expression in both monolayer and pellet cultures. Growth factors were selected based upon their previously demonstrated potential for articular cartilage, knee meniscus, and TMJ disk tissue engineering (22,24,25). Thus, this study aims to provide valuable insight into TMJ disk cellular changes caused by common tissue culture and tissue engineering practices.

Materials and methods

Harvest and culture conditions

Temporomandibular joint disks were isolated from female pigs weighing more than 61 kg (135 lbs) post-dressing; pigs in this weight range are skeletally mature (approximately 8–12 months old). All tissue was obtained from a local slaughterhouse. Five TMJ disks from five porcine heads were isolated for the purposes of examining growth factor effects on cells in monolayer and pellet culture. All disks were digested in 40 ml of 1 mg/ml collagenase type II (Worthington, Lakewood, NJ, USA) overnight. Cells were then plated on tissue culture plastic (TCP)-treated six-well plates at approximately 50% confluence and cultured in Dulbecco's modified eagle's medium with Glutamax (Gibco) containing 10% fetal bovine serum (Gemini), 25 μg/ml ascorbic acid (Sigma), 1% non-essential amino acids (Gibco), and 1% penicillin–streptomycin–fungizone (Biowhittaker). Full media changes were performed every other day.

Upon reaching confluence, cells were passaged by exposure to trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco). Cells were isolated from the trypsin using centrifugation (450 rpm for 5 min), and cell counts were obtained with a hemocytometer. Control samples were obtained at each passage by lysing cells in TriZol reagent. For the purposes of studying growth factor effects in monolayer, cells were plated on TCP-treated six-well plates (300 000 cells per well) and media were supplemented with either an additional 5 ng/ml of TGF-β3, 5 ng/ml of TGF-β1, or 10 ng/ml IGF-I. For the purposes of studying growth factor effects in pellet culture, cells were placed in a 0.5-ml Eppendorf tube (300 000 cells per tube) and centrifuged at 400 *g* for 2 min. Media were removed and growth factor supplemented media were added. Eppendorf lids were punctured for ventilation and placed in an enclosed sterile rack. All samples, monolayer and pellet, contained the same cell number based upon the initial hemocytometer count. Samples were cultured at 37°C; at 24 h, media were removed, and samples were lysed in TriZol reagent (Invitrogen, Carsbad, CA, USA).

RNA isolation and RT reaction

RNA was isolated using TriZol reagent, following the protocol provided by the manufacturer. Briefly, cells were vortexed in 1 ml TriZol, 0.2 ml of chloroform was mixed in each sample, and samples were centrifuged at 12 000 *g* for 15 min. The topmost, clear supernatant was isolated and moved to a fresh 1.5-ml Eppendorf tube, and 0.5 ml of isopropanol per original volume of TriZol was added to the supernatant. RNA was precipitated by centrifugation, washed with 1 ml of 75% ethanol, and dissolved in 40 μl of RNAse-free water. RNA concentration and purity were assessed on a spectrophotometer (Nanodrop, Wilmington, DE, USA).

RNA was reverse-transcribed to cDNA using a Stratascript^{TM} First Strand Synthesis System (Stratagene) according to the manufacturer's protocol. Briefly, 350 ng RNA per sample was suspended in 17.7 μl of RNAse-free water. As each reaction contained the same concentration of RNA and reagents, similar reverse transcription (RT) reaction efficiencies were assumed for all samples in the ensuing analyses. Random primers were annealed to the sample RNA by incubating samples with primers, buffer, and dNTPs at 65°C for 5 min followed by cooling to room temperature (approximately 10 min). The mixture was then incubated with RT and RNase block at 42°C for 60 min followed by reaction termination (70°C for 15 min).

PCR procedures

Real-time polymerase chain reaction (PCR) was performed for collagen type I, collagen type II, aggrecan, decorin, biglycan, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a Rotor-gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). HotStarTaq (Qiagen) combined with buffer (Qiagen, 10×), MgCl (Qiagen, 3.5 mM), dNTPs (Promega, 0.2 mM), RNAse-free water, and the gene-specific primer-probe sets were used as the PCR mix. The PCR reaction commenced with a 15-min denaturing step at 95°C followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. Fluorescence measurements (on FAM, CalRed, and Quasar 670, Biosearch Technologies, Novato, CA, USA) were taken every cycle at 60°C to provide a quantitative, real-time analysis of the PCR reaction for specific genes. The GAPDH primer-probe set was included in every PCR reaction as a verification gene. As GAPDH is abundant in TMJ disk cells, it is easily detectable in the PCR reactions, allowing samples with no expression to be separated from failed reactions. Primer-probe sequences for GAPDH are

available in Darling *et al.* (26); all other primer-probe sequences are available in Upton *et al.* (27).

Gene expression abundance

The efficiency (E) of the PCR reactions was determined by a series of standard sample dilutions (1×, 10×, 100×, and 1000×). Take-off cycle (Ct) for the standards and samples, where Ct is equal to the cycle at which 30% of the maximum slope for a PCR reaction occurs, was determined via the comparative quantification package available in the Rotogene software. Abundance values for the gene of interest (A_{GOI}) can be calculated for quantitative comparison from the following equation:

$$A_{GOI} = \frac{1}{(1 + E_{GOI})^{Ct_{GOI}}} \quad (1)$$

Abundances from three PCR reactions were examined to ensure that reaction loading was consistent. If consistent, their average was used for the ensuing statistical analysis; if not, the reaction was repeated. To ensure consistent reaction loading between PCR runs, the GAPDH results were compared, and if necessary, runs were repeated.

Statistical analysis

The distribution of abundances for each measured gene appeared nearly log-normal (Fig. 1). A log-normal distribution of gene expression data has previously been noted in single cells from the pancreatic islets of Langerhans (28) and also from single chondrocytes (A.C. Shieh, unpublished data). A logarithmic transformation of the original data set provides a distribution which more closely approximates the normal distribution; thus, abundances were transformed to their logarithmic equivalent prior to statistical analysis. A four-factor analysis of variance (ANOVA) approach was employed to analyze the significance of animal, passage, growth factor treatment, and pellet vs. monolayer culture. Animal, growth factor treatment, and pellet vs. monolayer culture were treated as categorical data; passage was considered continuous. Interactions were initially considered, but excluded from the final model because of a lack of significance. If an individual factor was significant in the ANOVA, a *post hoc* Tukey's HSD was conducted to investigate variations between treatment groups. In all statistical tests, a significance level of 0.05 was used.

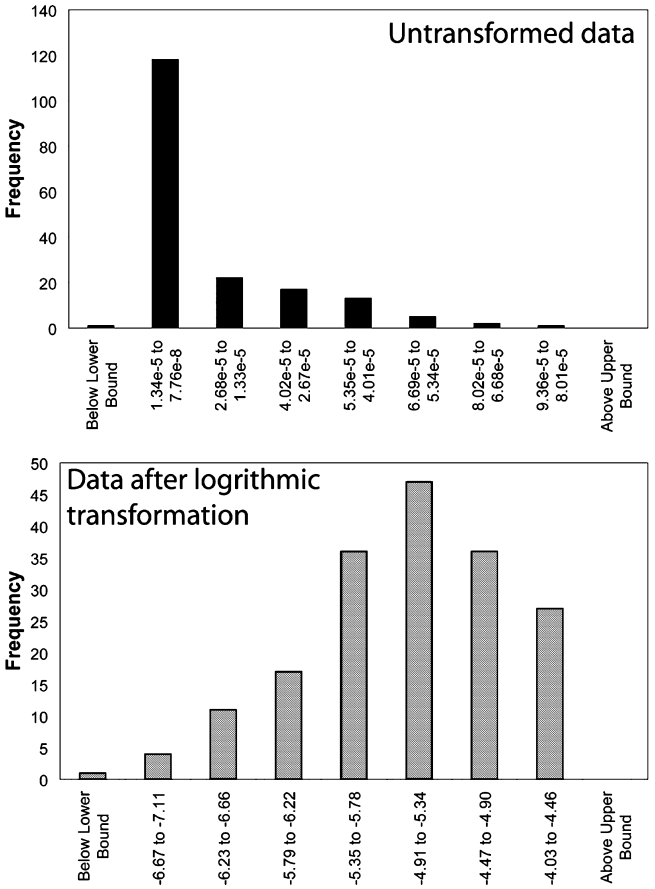

Fig. 1. Validation of log-normal normal distribution. The distribution of gene expression data, represented by a histogram (top), has a strong skew right and appears similar to that of a log-normal distribution. After a logarithmic transformation of the data, the histogram (bottom) more closely approximates that of a normal distribution, although maintaining a slight skew left. Because of the normality assumption within the statistical methods, the log-normal conversion provides a distinct advantage for our statistical analysis. The distribution of the collagen type I gene expression results is presented before and after the transformation. As can be verified by the figure, the transformation converted the response variable into an approximately normal distribution.

Results

Gene expression levels of collagen type I, decorin, biglycan, and aggrecan were lower in pellet culture compared with monolayer culture ($p < 0.005$, Fig. 2). Gene expression of collagen type I, the disk's primary ECM component, demonstrated the largest variations; monolayer cultures had collagen type I abundances that were 5.5 times higher than pellet cultures, on average. Aggrecan variations were also substantial, near

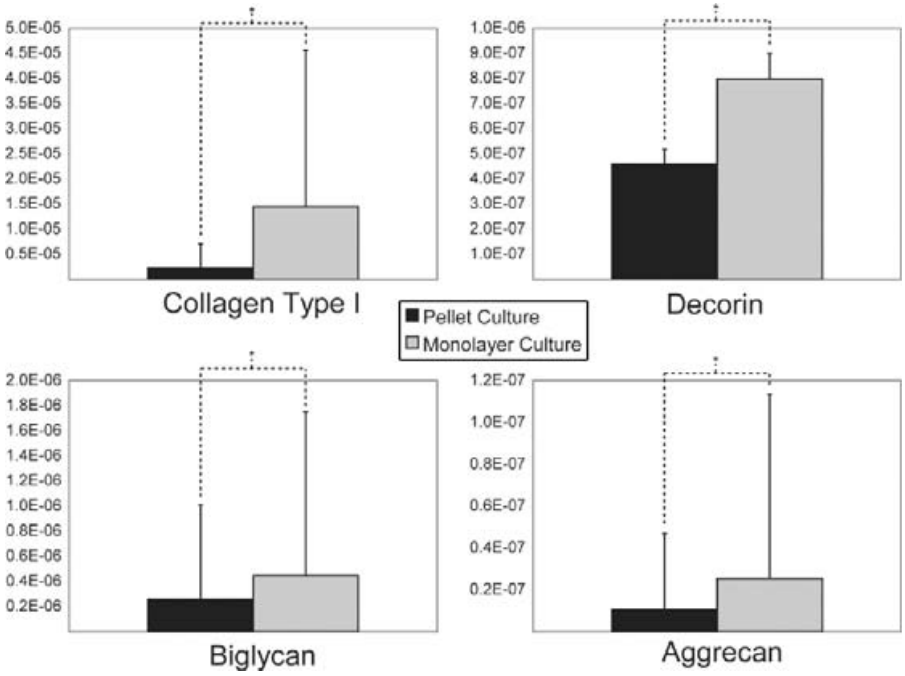

Fig. 2. Gene expression in monolayer culture vs. pellet culture for TMJ disk cells. Gene expression levels for collagen type I, decorin, biglycan, and aggrecan were lower in pellet cultures relative to monolayer cultures ($p < 0.005$). Mean and standard deviations for each gene were calculated from their logarithmic equivalents obtained from the statistical model. These data indicate that pelleting downregulates the gene expression of the TMJ disk's primary ECM proteins, suggesting that pelleting is a poor choice for TMJ disk tissue engineering.

1.4-fold, while decorin and biglycan variations were approximately 0.73-fold.

Similarly, pellet culture rapidly decreased collagen type I, aggrecan, and biglycan gene expression relative to passage controls (Figs 3 and 4). As cells were passaged, collagen type I and aggrecan levels in passaged controls and pellet cultures tended to converge; however, at early passages, substantial decreases in collagen type I and aggrecan gene expression were evident (Fig. 3). At passage 1, decreases were near 9.0- and 3.5-fold in collagen type I and aggrecan, respectively. Decreases in biglycan levels were consistent, near 3-fold at every passage examined (Fig. 4). Decorin abundances in pellet cultures were comparable with passage controls throughout the experiment (Fig. 4).

The mean level of decorin gene expression was higher in each growth factor group; these effects were especially apparent in monolayer cultures (Fig. 6). TGF-β3 significantly upregulated decorin gene expression relative to no-treatment controls ($p < 0.05$); decorin levels were 96% larger, on average, in TGF-β3 groups compared with no treatment. Growth factor did not have a significant effect on any other measured gene; however, the trends for collagen type I expression and biglycan expression were identical (Table 1). Furthermore, the mean value of aggrecan gene expression was higher in each treatment group relative to no treatment controls.

Differences between passage controls and monolayer culture samples were not as apparent (Figs 5 and 6). At passage 1, collagen type I gene expression was lower in monolayer cultures relative to passage controls; however, at later passages collagen type I gene expression was similar to passage controls (Fig. 5). Aggrecan levels in monolayer culture relative to passage controls were more irregular. Generally, aggrecan levels were lower in monolayer culture relative to passaged control and growth factors had little effect on aggrecan expression (Fig. 5). Decorin gene expression tended to be larger in monolayer cultures than passaged cultures, especially those presented with a growth factor (Fig. 6). Biglycan levels in monolayer culture were lower than passaged control, especially those cultures not treated with a growth factor treatment; thus, the presence of a TGF-β1 and TGF-β3 demonstrated some ability to return biglycan levels near that of passage controls (Fig. 6).

Passage effects on TMJ disk cells were similar to those reported previously (19). Collagen type I and aggrecan expressions were rapidly downregulated with passage with predicted decreases of 28% and 44% per passage, respectively ($p < 0.0001$). Collagen type II expression was undetectable after the second passage. Decorin expression was predicted to increase by 10% per passage. This increase was not significant ($p = 0.12$), but is within the predicted range of the increase reported previously (19). Biglycan expression decreased 16% per passage ($p < 0.01$). The PCR response for collagen type II was very low relative to other genes. In addition, collagen type II gene

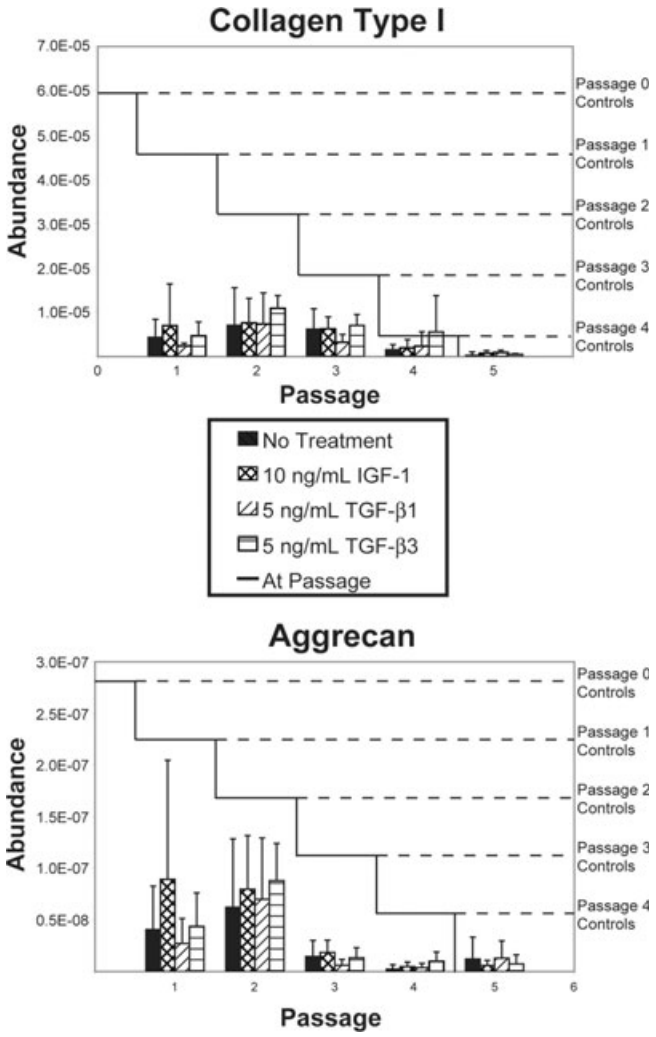

Fig. 3. Growth factor effects in pellet culture (Collagen Type I and Aggrecan). Passage controls are represented by the solid black line; no treatment controls at each passage are represented by a solid black bar. Collagen type I and aggrecan gene expression levels dropped rapidly due to pelleting. These losses were not recovered by exposure to a growth factor. Losses in collagen type I were the most significant; as early as passage 1, pelleting resulted in a 9.0-fold reduction of collagen type I gene expression. Aggrecan losses were also substantial at early passages with a 3.5-fold reduction at passage 1. Losses in aggrecan and collagen type I became less substantial with passage, primarily because of decreases in the passage controls.

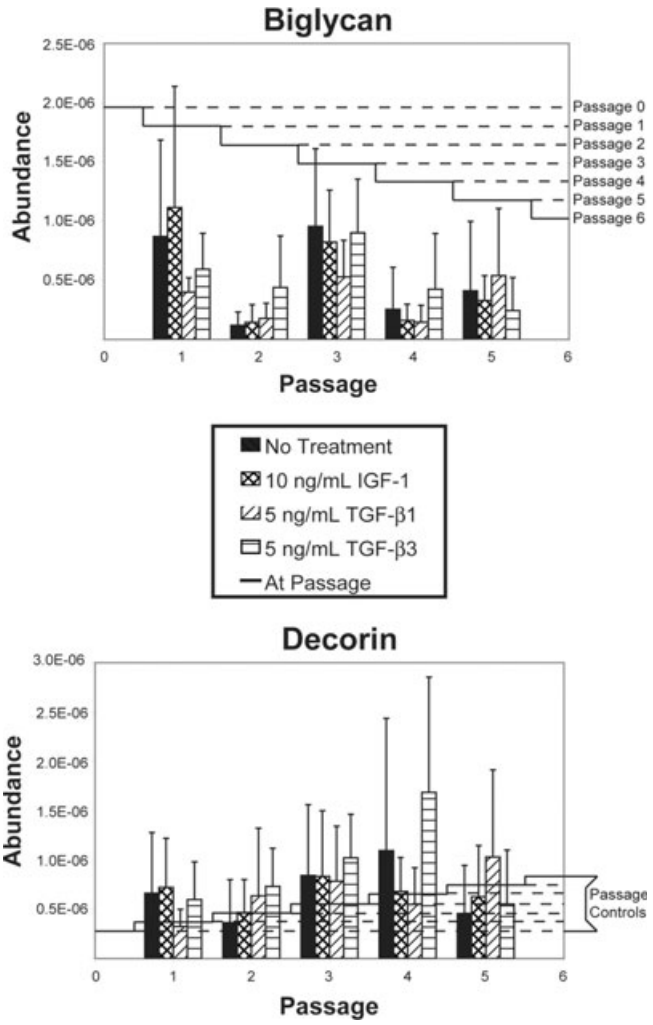

Fig. 4. Growth factor effects in pellet culture (Decorin and Biglycan). Passage controls are represented by the solid black line; no treatment controls at each passage are represented by a solid black bar. Biglycan levels dropped rapidly because of pellet culture and were consistently lower in pellet cultures relative to passage controls. Biglycan reductions were near 3-fold at every examined passage; these losses could not be recovered by exposure to a growth factor. Decorin expression was near passaged controls at every passage.

expression levels were rarely detected after passage 2. As a result, many singularities in the statistical model were created, making analysis of collagen type II changes impossible. Regardless, collagen type II gene expression was near zero or negligible in most samples.

Discussion

Pellet cultures have demonstrated potential for articular cartilage tissue engineering (29–31). Based upon substantial down-regulation of TMJ disk ECM gene expression, pellet culture appears to be a poor choice for TMJ disk tissue engineering. In pellet cultures, collagen type I, aggrecan, and biglycan gene expression was rapidly down-regulated relative to both monolayer cultures and passaged controls; in addition, decorin gene expression was lower in pellet cultures relative to monolayer cultures. This down-regulation of ECM genes indicates a decrease in potential for ECM deposition, suggesting a significant decrease in potential for tissue engineering success in pellet cultures. Of greatest concern is the inability to rescue collagen type I expression by placing TMJ disk cells in a pellet

Table 1. **Growth factor effects on TMJ disk gene expression**

	Collagen type 1					Decorin				
	Low		Mean		High	Low		Mean		High
TGF-β3	2.6E-06	←	1.2E-05	→	2.0E-05	3.0E-07	←	1.4E-06	→	2.4E-06
TGF-βI	5.9E-06	←	1.1E-05	→	1.6E-05	6.9E-07	←	1.3E-06	→	2.0E-06
IGF-I	5.9E-06	←	1.1E-05	→	1.6E-05	5.4E-07	←	1.0E-06	→	1.5E-06
No treatment	4.2E-06	←	7.9E-06	→	1.2E-05	4.3E-07	←	8.3E-07	→	1.2E-06
	Biglycan					Aggrecan				
TGF-β3	1.3E-07	←	8.3E-07	→	1.5E-06	5.2E-09	←	4.0E-08	→	7.4E-08
TGF-βI	3.7E-07	←	8.1E-07	→	1.3E-06	1.5E-08	←	3.5E-08	→	5.6E-08
IGF-I	3.2E-07	←	7.1E-07	→	1.1E-06	1.9E-08	←	4.5E-08	→	7.1E-08
No treatment	2.5E-07	←	5.4E-07	→	8.4E-07	1.3E-08	←	3.2E-08	→	5.0E-08

The mean and ranges for each gene were converted from their logarithmic equivalent as calculated by the statistical model. TGF-β3 produced the highest mean of collagen type I, decorin, and biglycan gene expression, followed by TGF-β1, IGF-I, then no treatment. All growth factor treatments also had a higher mean for aggrecan gene expression relative to no treatment. These variations, however, were not statistically significant, with one exception; decorin expression was significantly larger in TGF-β3 treated groups relative to no treatment.

environment or exposing them to a growth factor. The TMJ disk is almost entirely collagen type I. Inability to produce this particular protein is detrimental to tissue engineering success using either *in vitro* or *in vivo* techniques. In addition, aggrecan, the disk's primary proteoglycan (32), could not be fully rescued by exposure to TGF-β3, TGF-β1, or IGF-I. These growth factors have previously demonstrated a potential for increasing ECM deposition in cartilage tissue engineering (21–24,33); however, their effectiveness on TMJ disk gene expression was marginal in pellet cultures.

The inability to retain or recover gene expression for TMJ disk ECM matrix is of concern for procedures using autologous cells for implantation. At minimum, implanted cells must produce a tissue which restores the volume, geometry, and mechanical integrity of the joint; ideally, these neo-tissues would possess matching mechanical and biochemical properties as well. The use of autologous chondrocytes for implantation in the knee has demonstrated positive effects (13), yet fibrous tissue formation at the graft site may ensue (34). The dedifferentiation of articular chondrocytes because of *in vitro* expansion has been demonstrated via an RT-PCR analysis; as chondrocytes are passaged, chondrocytic markers are down-regulated while fibroblastic markers are up-regulated (18). Thus, these data support the finding of fibrous tissue formation at autologous chondrocyte implant graft sites. In our analysis of passaged TMJ disk cells, critical ECM components are down-regulated during passaging, further reduced by pelleting, and not readily recovered via exposure to IGF-I, TGF-β1 or TGF-β3. Thus, it is not clear that passaged autologous TMJ disk cells will form a neo-tissue quickly or with acceptable biochemical components.

Cultures exposed to growth factors generally had a higher abundance of ECM gene expression. Although differences were not statistically significant, TGF-β3 had the highest mean value for collagen type I, decorin, and biglycan expression, followed by TGF-β1 and IGF-I; growth factor-exposed groups also had larger means for aggrecan gene expression. The effects of growth factors were minimal relative to the effects of passage and pelleting. However, the positive effects of growth factors for TMJ disk tissue engineering cannot be ruled out; however, these positive effects are likely overwhelmed in pellet cultures. Furthermore, the cellular pathways toward protein production are lengthy with many checks and balances, and while exposure to a growth factor may not induce a dramatic upregulation at the gene expression level, other parts of the production pathway such as the translation of mRNA transcripts to protein code may be positively affected.

Gene expression levels in monolayer cultures did not vary from passaged controls as substantially as pellet cultures; however, a few variations are of note. Biglycan

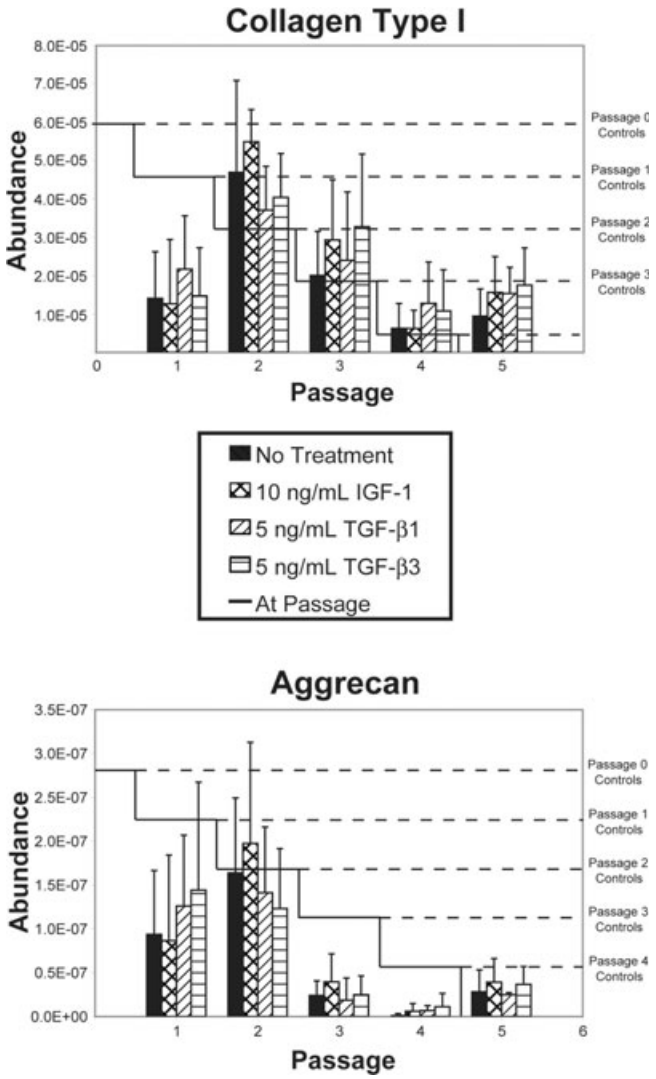

Fig. 5. Growth factor effects in monolayer culture (Collagen Type I and Aggrecan). Passage controls are represented by the solid black line; no treatment controls at each passage are represented by a solid black bar. Collagen type I gene expression was lower at passage 1; however, at later passages, collagen type I expression was comparable with passage controls. Aggrecan gene expression tended to be lower than passage controls at each passage; however, fold variations varied with passage.

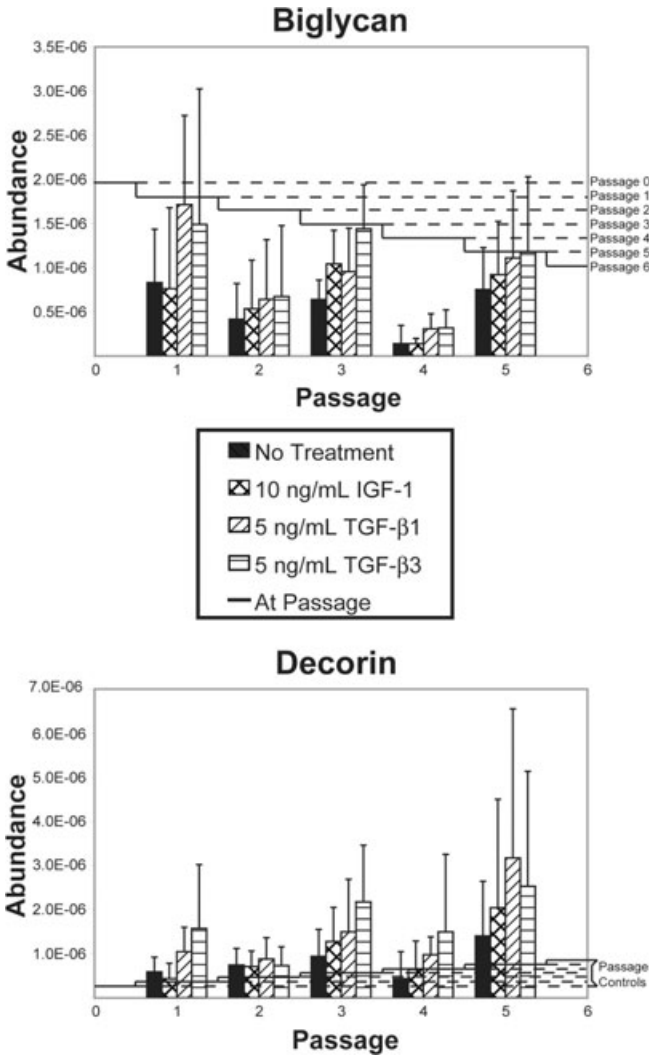

Fig. 6. Growth factor effects in monolayer culture (Decorin and Biglycan). Passage controls are represented by the solid black line; no-treatment controls at each passage are represented by a solid black bar. Biglycan gene expression tended to be lower than passage controls; however, biglycan expression rebounded at many passages by exposing the culture to TGF-β3 or TGF-β1. Similar upregulation was seen in decorin; at most passages, decorin gene expression was up-regulated by exposure to a growth factor. TGF-β3 had the greatest effects and showed a significant difference from no-treatment control in decorin expression ($p < 0.05$).

levels were lower in monolayer culture than passaged controls; however, biglycan levels could be partially recovered with TGF-β3 or TGF-β1. Decorin, a proteoglycan similar to biglycan, was also up-regulated in cultures exposed to TGF-β3 or TGF-β1. This decorin upregulation, however, placed decorin gene expression levels much higher than passaged controls. Thus, the rescue of biglycan expression may also induce overexpression of decorin.

The effect of passage on TMJ disk cells was similar to those described previously (19). Collagen type I, collagen type II, and aggrecan dropped rapidly with passage. The observed decrease in aggrecan and collagen type I was more rapid in this experiment than our previous results, but the predicted decrease for both genes is within the error of our earlier data. Decorin trended upward with passage, similar to our earlier findings; however, increases were far less substantial in the present experiment and not statistically significant. Biglycan, on the other hand, showed a significant decrease with passage. Previously, this trend was noted, but not considered significant. These data on passage effects follow the same trends as our previous report,

and the predicted increases and decreases fall within the error of our previous report. The effect of passage on TMJ disk cells is substantial and should be accounted for in experiments using passaged TMJ disk cells.

To passage cells, the culture is typically exposed to a digestive enzyme such as trypsin/EDTA. Cells are then removed, centrifuged, and re-suspended in a new culture. Previously, we attributed changes in gene expression to exposure to a digestive enzyme (19); however, our pellet culture data suggest that centrifugation may have equal or more importance. Centrifugation imparts a mechanical stimulus on the entirety of the cell mass. This stimulus may lead to changes in gene expression via mechanotransduction pathways. Mechanotransduction is a powerful stimulus on cartilaginous tissues. Intermittent hydrostatic pressure has caused detrimental effects on TMJ disk cells (35); sustained direct compression has negatively affected knee meniscus and articular cartilage explants (36–37). Thus, even the simplest mechanical stimuli applied during culture and tissue engineering practices may have large effects.

For many of our samples, collagen type II was not detectable at any passage; by passage 3, collagen type II expression fell below the level of detection in all samples. Relative to collagen type I, collagen type II is a minor ECM constituent in the TMJ disk; however, its expression is indicative of chondrocyte-like properties of TMJ disk cells and hyaline-like activity. Its initial low level and rapid decrease demonstrates the dominance of fibroblast-like cells in the TMJ disk. Furthermore, failure to rescue collagen type II may indicate an inability to recapitulate hyaline-like matrix in a tissue engineered TMJ disk. While pockets of collagen type II are sparse in the TMJ disk, creating these structures may be important in TMJ disk function. Thus, in future tissue engineering studies, the lack of chondrocytic potential in TMJ disk cells may need to be addressed.

Gene expression of the TMJ disk's primary ECM proteins decreased with passage with the exception of decorin. These losses were not recovered in pellet culture; in fact, pelleting generally led to further losses in expression of aggrecan, biglycan, and collagen type I. In addition, losses were not rescued by supplementing the pellet culture media with growth factors. In monolayer, the effects of growth factors were more apparent. Gene expression of dermatan sulfate proteoglycans, decorin and biglycan, appeared to be up-regulated by TGF-β1 and TGF-β3. Collagen type I gene expression was also generally higher in growth factor groups. These effects, however, were far less substantial than passaging or pelleting effects, and were often not statistically significant. Our results indicate that pellet cultures are a poor choice for TMJ disk tissue engineering. In addition, growth factors at the investigated concentrations are unable to rescue essential TMJ disk gene expression.

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References

1. Wilkes CH. Internal derangements of the temporomandibular joint. Pathological variations. *Arch Otolaryngol Head Neck Surg* 1989;**115**:469–77.
2. Stohler CS. Taking stock: from chasing occlusal contacts to vulnerability alleles. *Orthod Craniofac Res* 2004;**7**:157–61.
3. Carlsson GE, Kopp S, Lindstrom J, Lundqvist S. Surgical treatment of temporomandibular joint disorders. A review. *Swed Dent J* 1981;**5**:41–54.
4. Gillbe GV. The function of the disc of the temporomandibular joint. *J Prosthet Dent* 1975;**33**:196–204.
5. Kondoh T, Hamada Y, Kamei K, Seto K. Simple disc reshaping surgery for internal derangement of the temporomandibular joint: 5-year follow-up results. *J Oral Maxillofac Surg* 2003;**61**:41–8. discussion 48.
6. Clark GT, Seligman DA, Solberg WK, Pullinger AG. Guidelines for the treatment of temporomandibular disorders. *J Craniomandib Disord* 1990;**4**:80–8.
7. Feinberg SE. Use of local tissues for temporomandibular joint surgery disc replacement. *Atlas Oral Maxillofac Surg Clin North Am* 1996;**4**:51–74.
8. Detamore MS, Athanasiou KA. Motivation, characterization, and strategy for tissue engineering the temporomandibular joint disc. *Tissue Eng* 2003;**9**:1065–87.
9. FDA. *FDA Enforcement Report [FDA Report]*. Rockville: United States Food and Drug Administration; 1991.
10. Fontenot MG, Kent JN. In vitro wear performance of Proplast TMJ disc implants. *J Oral Maxillofac Surg* 1992;**50**:133–9.
11. Henry CH, Wolford LM. Treatment outcomes for temporomandibular joint reconstruction after Proplast-Teflon implant failure. *J Oral Maxillofac Surg* 1993;**51**:352–8. discussion 359–60.
12. Allen KD, Athanasiou KA. Tissue engineering the temporomandibular joint disc. *Tissue Eng* 2006;**12**:1183–96.
13. Peterson L, Brittberg M, Kiviranta I, Akerlund EL, Lindahl A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am J Sports Med* 2002;**30**:2–12.
14. Caterson EJ, Nesti LJ, Albert T, Danielson K, Tuan R. Application of mesenchymal stem cells in the regeneration of musculoskeletal tissues. *MedGenMed (Electronic Source)* 2001;E1.
15. Semb H. Human embryonic stem cells: origin, properties and applications. *APMIS* 2005;**113**:743–50.
16. Risbud MV, Shapiro IM. Stem cells in craniofacial and dental tissue engineering. *Orthod Craniofac Res* 2005;**8**:54–9.

17. Almarza AJ, Athanasiou KA. Effects of initial cell seeding density for the tissue engineering of the temporomandibular joint disc. *Ann Biomed Eng* 2005;**33**:943–50.
18. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res* 2005;**23**:425–32.
19. Allen KD, Athanasiou KA. Effect of passage and topography on gene expression of TMJ disc cells. *Tissue Eng* 2006 (in press).
20. Detamore MS, Hegde JN, Wagle RR, Almarza AJ, Montufar-Solis D, Duke PJ et al. Cell type and distribution in the porcine temporomandibular joint disc. *J Oral Maxillofac Surg* 2005;**64**:243–8.
21. Detamore MS, Athanasiou KA. Effects of growth factors on temporomandibular joint disc cells. *Arch Oral Biol* 2004;**49**:577–83.
22. Detamore MS, Athanasiou KA. Evaluation of three growth factors for TMJ disc tissue engineering. *Ann Biomed Eng* 2005;**33**:383–90.
23. Pangborn CA, Athanasiou KA. Effects of growth factors on meniscal fibrochondrocytes. *Tissue Eng* 2005;**11**:1141–8.
24. Pangborn CA, Athanasiou KA. Growth factors and fibrochondrocytes in scaffolds. *J Orthop Res* 2005;**23**:1184–90.
25. Roark EF, Greer K. Transforming growth factor-beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. *Dev Dyn* 1994;**200**:103–16.
26. Darling EM, Hu JC, Athanasiou KA. Zonal and topographical differences in articular cartilage gene expression. *J Orthop Res* 2004;**22**:1182–7.
27. Upton ML, Chen J, Guilak F, Setton LA. Differential effects of static and dynamic compression on meniscal cell gene expression. *J Orthop Res* 2003;**21**:963–9.
28. Bengtsson M, Stahlberg A, Rorsman P, Kubista M. Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels. *Genome Res* 2005;**15**:1388–92.
29. Tare RS, Howard D, Pound JC, Roach HI, Oreffo RO. Tissue engineering strategies for cartilage generation-micromass and three dimensional cultures using human chondrocytes and a continuous cell line. *Biochem Biophys Res Commun* 2005;**333**:609–21.
30. Lubke C, Ringe J, Krenn V, Fernahl G, Pelz S, Kreusch-Brinker R et al. Growth characterization of neo porcine cartilage pellets and their use in an interactive culture model. *Osteoarth Cart* 2005;**13**:478–87.
31. Zhang Z, McCaffery JM, Spencer RG, Francomano CA. Hyaline cartilage engineered by chondrocytes in pellet culture: histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants. *J Anat* 2004;**205**:229–37.
32. Detamore MS, Orfanos JG, Almarza AJ, French MM, Wong ME, Athanasiou KA. Quantitive analysis and comparative regional investigation of the extracellular matrix of the porcine temporomandibular joint disc. *Matrix Biol* 2005;**24**:45–57.
33. Almarza AJ, Athanasiou KA. Evaluation of three growth factors in combinations of two for TMJ disc tissue engineering. *Arch Oral Biol* 2006;**51**:215–21.
34. Tins BJ, McCall IW, Takahashi T, Cassar-Pullicino V, Roberts S, Ashton B et al. Autologous chondrocyte implantation in knee joint: MR imaging and histologic features at 1-year follow-up. *Radiology* 2005;**234**:501–8.
35. Almarza AJ, Athanasiou KA. Effects of hydrostatic pressue on TMJ disc cells. *Tissue Eng* 2006;**12**:1285–94.
36. Ragan PM, Badger AM, Cook M, Chin VI, Gowen M, Grodzinsky AJ et al. Down-regulation of chondrocyte aggrecan and type-II collagen gene expression correlates with increases in static compression magnitude and duration. *J Orthop Res* 1999;**17**:836–42.
37. Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res* 1989;**7**:619–36.