Optimization of the Expansion and Differentiation of Rabbit Chondrocytes *In Vitro*

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Abstract

Objective: To develop a tissue culture expansion method for rabbit chondrocytes that promotes robust expansion while preserving chondrogenic potential. *Design:* Rabbit chondrocytes isolated from articular or auricular chondrocytes were assessed for chondrogenic differentiation potential versus population doubling using different expansion and differentiation conditions. Expansion conditions included serum alone, serum plus basic fibroblast growth factor 2 (FGF-2), and serum plus insulin-like growth factor 1 (IGF-1) and FGF-2. Differentiation conditions consisted of defined medium with and without bone morphogenetic protein 2 (BMP-2). *Results:* Nonsupplemented chondrocytes showed limited expandability, whereas supplementation with FGF-2 allowed articular chondrocytes to be expanded past 10 population doublings (PDs) and allowed auricular chondrocytes to expand past 15 population doublings. Differentiation, as measured by glycosaminoglycan production in aggregate cultures, was minimal in articular chondrocytes without BMP-2 supplementation and diminished to less than 50% maximal in auricular chondrocytes by PD 20. However, when FGF-2 was used during expansion and BMP-2 used during differentiation, both articular and auricular chondrocytes retained greater than 50% maximal differentiation for more than 25 PDs. The addition of IGF-1 to FGF-2 during expansion decreased chondrogenicity of auricular chondrocytes exposed to BMP-2, whereas for articular chondrocytes, chondrogenic expression increased. *Conclusion:* These results demonstrate that FGF-2, for expansion, and BMP-2, for differentiation, dramatically increase the functional expansion of auricular and articular chondrocytes and provide a methodology to expand sufficient numbers of chondrocytes for tissue engineering applications.

Keywords

cartilage, growth factors, tissue engineering, tissue culture

Introduction

Two major studies in this laboratory are dependent on the use of culture-expanded chondrocytes that are then applied to the development of tissue-engineered trachea or joint tissues.[1-5] A major limitation in these studies is the loss of chondrogenic potential during culture expansion. This culture expansion limitation has been recognized by several other laboratories[6-9] wherein chondrocytes were shown to lose re-differentiation potential after only a few cell passages. A multitude of studies have investigated culture conditions that promote chondrocyte cell expansion and subsequent re-differentiation into chondrocytes, but only a few studies have been on chondrocytes isolated from rabbit tissues, and even fewer of these studies have looked at a combination of expansion growth factors and differentiation factors being used in concert. For example, bone morphogenetic protein 2 (BMP-2) added to rabbit articular chondrocytes showed modest increases in aggrecan levels,[10] but the study was conducted on monolayer cultures, which are not amenable to chondrocyte differentiation as are conditions that promote

rounding of cells and cell-to-cell interactions.[11] Transforming growth factor β (TGFβ) and hyaluronic acid[12] were shown to increase glycosaminoglycan (GAG) production in rabbit articular chondrocytes compared to medium only or medium plus hyaluronic acid, and another study showed that TGFβ2 was stimulatory to GAG production in alginate cultures,[13] but no cell expansion studies were performed in either of these studies. Only a few studies investigated the use of growth factors for expansion and differentiation of rabbit chondrocytes. Van Osch *et al.*[14] showed some improvement in rabbit nasal septum chondrocyte differentiation after culture expansion in medium containing TGFβ2 and insulin-like growth

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factor 1 (IGF-1), but again, no combination of growth factors during expansion and then during differentiation was reported. One of the first studies to use a defined medium for the expansion and differentiation of chondrocytes showed positive effects of IGF-1 and basic fibroblast growth factor 2 (FGF-2) in rabbit chondrocytes,[15] which highlighted both IGF-1 and FGF-2 as likely candidate molecules for our study.

Clearly, there is a growing need for methods to expand chondrocytes while retaining differentiation potential for the application of these cells to construct large engineered tissues, such as for reconstructing the trachea.[4] The goal of this study was to conduct an initial screen for candidate growth factors for rabbit chondrocyte expansion and, separately, to identify candidates for differentiation and, finally, to put together these candidate molecules and test the retention of chondrocyte differentiation capacity after extended culture using the expansion and differentiation growth factors in concert. Our results demonstrate that a combined use of FGF-2 during expansion, along with the application of BMP-2 during differentiation, not only can extend the absolute number of chondrocyte population doublings but can, more importantly, significantly increase the number of differentiation-capable cells up to 20 population doublings (PDs) or more. These results make studies on the development of large tissue-engineered cartilage constructs in rabbits much more feasible.

Methods

Cell Culture: Chondrocyte Expansion

Articular and auricular cartilage was harvested from 3 skeletally mature New Zealand White rabbits, creating 6 independent isolates—3 each of articular- and auricular-derived cells. Chondrocytes from each of the 6 isolates were divided into 5 separate expansion conditions (30 total independent states), with each condition plated onto a T-75 flask at an initial seeding density of 3,300 cells/cm^2 (approximately 250,000 cells per flask). Four of the 5 expansion conditions to be compared were set in a basal medium of Dulbecco's modified Eagle's medium (DMEM) with 1 g/L glucose and supplemented with 5% fetal bovine serum (FBS), with the fifth condition set in 10% FBS and DMEM (with 1 g/L glucose) alone. Three of 5 groups were placed in 1 of 3 conditions: FGF-2 (10 ng/mL), IGF-1 (100 ng/mL), or FGF-2 and IGF-1 in combination. The concentrations selected for FGF-2 and IGF-1 testing were based on prescreening results (data not shown). Two of the 5 groups consisted of either 5% FBS or 10% FBS with DMEM without the addition of growth factors. All media were supplemented with 10,000 U/mL penicillin G and 10,000 mg/mL streptomycin sulfate. Cells were cultured in 5% CO_2 at 37°C using a standard CO_2-control cell culture incubator. The media in each flask were exchanged 2 times per week, and flasks were trypsinized and passaged at confluence (end of

P^0) and subsequently at P^n; cells were counted for determination of doublings and replated at 1 million cells per flask. Cultures were passaged based on confluence rather than time because the differences in growth rates in different conditions would have resulted in over- or undercrowding of cells in different growth conditions, which could then affect growth or differentiation characteristics.

Microaggregate Culture: Chondrocyte Differentiation

The microaggregate culture system used to assay for chondrogenesis was that described by Yoo *et al*.[11] and modified for 96-well plates.[16] Following trypsinization at passages 1, 3, 5, 7, and 9, aliquots from each group were plated onto a 96-well plate for the formation of microaggregates. Four microaggregates, consisting of 250,000 cells per aggregate, from each group were cultured in 200 μL aggregate medium (AM; DMEM + 10^{-7} M dexamethasone, 1% ITS [Invitrogen, Carlsbad, CA] and 37.5 μg/mL ascorbate-2-phosphate) and 4 in AM + BMP-2 at 50 ng/mL. Although the initial screen for BMP-2 was performed at 100 ng/mL, a subsequent dilution experiment showed that 50 ng/mL was just as effective at increasing GAG accumulation (data not shown). The aggregate media were changed 3 times per week, and aggregates were maintained for 3 weeks prior to harvest. At harvest, all aggregates were weighed with one of the four prepared for histology and the other three analyzed for DNA and GAG content, as described.[2] Histology was performed with the use of Safranin O with Fast Green counterstain.
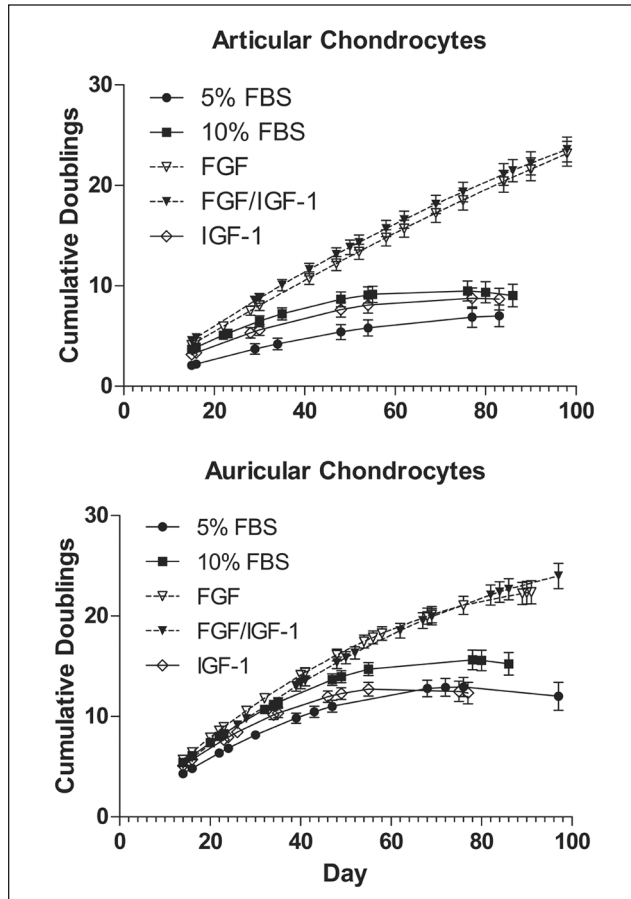
Statistical Methods

A mixed-model growth curve analysis was used to estimate and compare quadratic curves of cumulative doublings versus day, up to day 100 for each combination of the 5 expansion conditions and 2 media (BMP-2 or AM), where separate analyses were carried out for each cartilage type. The mixed model was fit using SAS Proc Mixed and included random effects for the intercept and linear terms that were specific to rabbit, as well as to expansion condition within rabbit, to account for two levels of correlation (within rabbit and within the same expansion culture over time). Contrasts were used to test whether the entire curves differed between groups of interest. A similar mixed model was used to estimate and compare quadratic growth curves of GAG content versus cumulative doublings.

Results

Prescreening of Mitogens/Growth Factors

Because of the large number of samples and extended culture times needed to assess long-term retention of chondrogenic

Figure 1. A mixed-model growth curve analysis was used to fit and compare quadratic curves of cumulative doublings versus day between the 5 expansion conditions. The growth curves for FGF and FGF/IGF-1-supplemented cultures were significantly different from the other groups at $\rho < 0.05$. FBS = fetal bovine serum; IGF-1 = insulin-like growth factor 1; FGF = fibroblast growth factor.

potential, an initial screening for chondrocyte expansion was first conducted in medium supplemented with TGFβ, IGF-1, platelet-derived growth factor (PDGF), and FGF-2, including IGF-1, TGFβ, and PDGF in combination with FGF (**Suppl. Fig. S1**). Although not intended to be definitive, these results suggested that FGF-2 was the most mitotic, followed by PDGF and IGF-1. By itself, TGFβ1 seemed to inhibit chondrocyte expansion, especially in articular chondrocytes. From this initial screen, FGF-2 was selected for detailed analysis; TGFβ was eliminated, whereas IGF-1 and PDGF were indistinguishable from each other. On the basis of other published results on the use of IGF-1 in chondrocyte expansion[15] and on these preliminary results, IGF-1 was also selected for detailed analysis.

Several factors were tested for their purported chondrogenic inductive capacity, including dexamethasone, thyroxine (T3), and several members of the TGFβ superfamily (TGFβ1, TGFβ3, and BMPs -2, -6, and -7). The GAG assayed indicated that all 3 BMPs (2, 6, and 7) were capable of promoting GAG accumulation, whereas dexamethasone, T3, and TGFβ1 had a minimal positive effect (**Suppl. Fig. S2**).
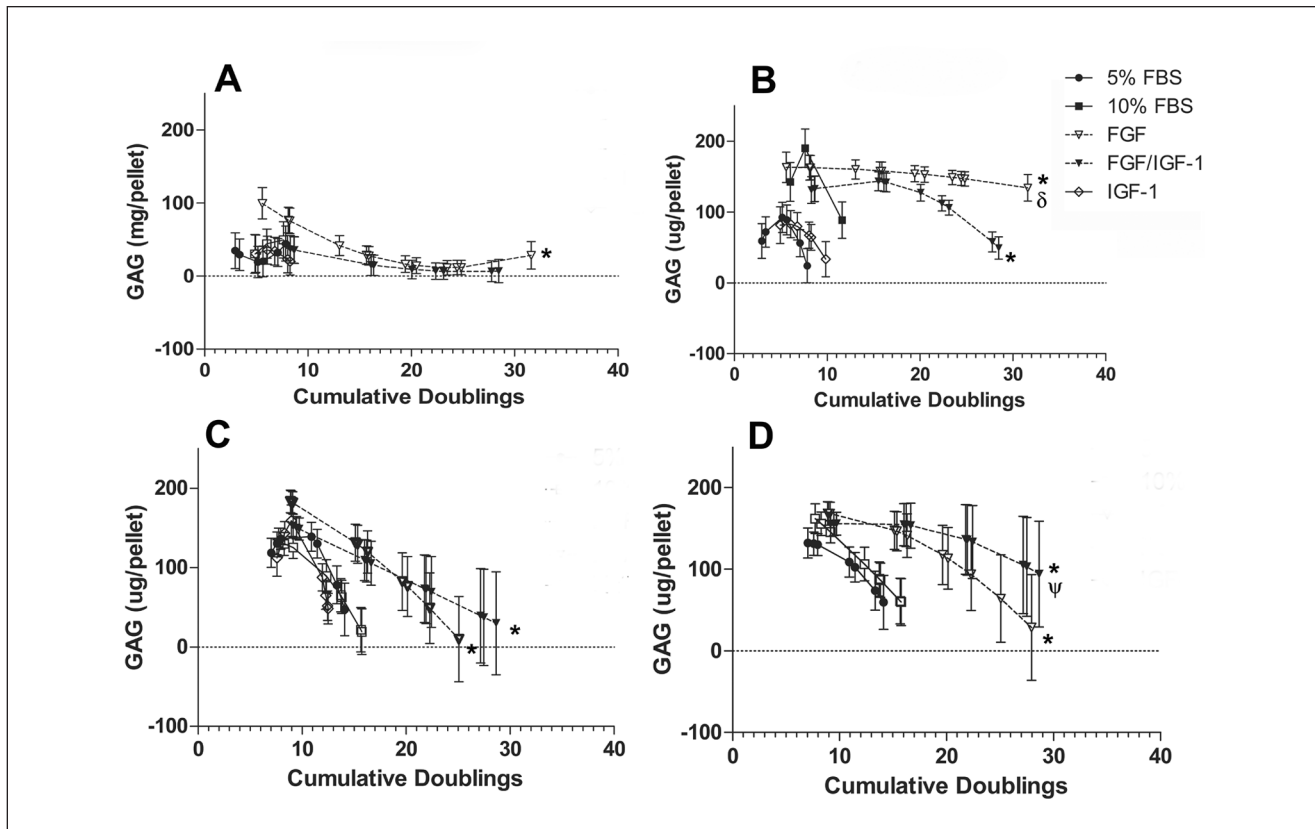
Cumulative Population Doublings

Estimated curves of mean cumulative doublings versus day for articular- and auricular-derived chondrocytes are shown in **Figure 1**. The significant similarity between the results obtained from different rabbit chondrocyte sources allowed the grouping of the means from 3 separate experiments, and this quadratic fit is what is plotted in **Figure 1**. The curves are termed *predicted values* as these are quadratic curves fit to the mean values of doublings over time. All comparisons are significantly different from each other ($\rho < 0.05$) except those between the 10% FBS, 5% FBS, and IGF-1 groups for auricular and articular aggregates, as well as between FGF-2 and FGF-2 + IGF-1 (F/I) conditions in articular aggregates. Both auricular and articular chondrocytes showed continued expansion past 90 days in medium containing FGF-2, whereas serum only or 5% serum plus IGF-1 showed significantly less cell expansion. Without FGF-2 present, articular chondrocytes did not expand more than 9 PDs, and auricular chondrocytes did not expand more than 15 PDs. With FGF-2 present, both articular and auricular chondrocytes expanded past 20 PDs.

Chondrogenesis versus Cell Expansion

To assess the chondrogenic potential of expanded chondrocytes, microaggregates were analyzed and compared for GAG content. As for PD analysis, a mixed-model growth curve analysis was used to fit quadratic curves to a grouping of means. For articular cartilage aggregates cultured in AM alone, there was a significant increase in GAG production for those expanded in FGF-2 versus 10% FBS, 5% FBS, and IGF-1 (**Fig. 2A**). For those aggregates cultured in AM + BMP-2, there was a significant increase in GAG production for those expanded in FGF-2 or FGF-2/IGF-1 versus 10% FBS, 5% FBS, or IGF-1 (**Fig. 2B**). For auricular aggregates incubated in AM without BMP-2 (**Fig. 2C**), there was a significant increase in GAG production for those expanded in FGF-2 versus 10% FBS, 5% FBS, and 5% FBS plus IGF-1, as well as for those expanded in FGF-2/IGF-1 versus 10% FBS and 5% FBS plus IGF-1. For auricular aggregates grown in AM + BMP-2 (**Fig. 2D**), there was a significant increase in GAG production for those expanded in FGF when compared with 5% FBS and 5% FBS + IGF-1 and for FGF-2/IGF-1 when compared with 5% or 10% FBS or 5% FBS + IGF-1.

When comparing aggregates grown in AM versus those grown in AM + BMP-2, there was a significant increase in

Figure 2. A mixed-model growth curve analysis used to fit and compare quadratic curves of mean glycosaminoglycan (GAG) content versus cumulative doublings for the 5 expansion conditions. Articular chondrocytes are shown (**A**) without and (**B**) with the addition of bone morphogenetic protein 2 (BMP-2). Auricular chondrocytes are shown (**C**) without and (**D**) with the addition of BMP-2. Basic fibroblast growth factor 2 (FGF-2) increases chondrogenic potential of both articular and auricular chondrocytes after expansion even without BMP-2 present (**A, C**). However, when BMP-2 is present, the effect of FGF-2 during expansion is even more dramatic (**B, D**). * indicates significant difference, at $\rho < 0.05$, from 5% FBS, 10% FBS, and IGF-1 groups; δ indicates significant difference from FGF/IGF-1; ψ indicates significant difference from FGF group. FBS = fetal bovine serum; IGF-1 = insulin-like growth factor 1; FGF = fibroblast growth factor.

GAG production across all expansion conditions, with the exception of auricular aggregates expanded in 5% or 10% FBS. In all cases, supplementation with IGF-1 alone did not significantly increase GAG production over that of serum-alone conditions, except for in BMP-2-supplemented conditions of articular chondrocytes, but even that increase dropped precipitously by PD 10.

Auricular and articular chondrocytes exhibited significant differences in baseline differentiation characteristics. Auricular chondrocytes produced greater amounts of GAG per aggregate in all expansion conditions than did articular chondrocytes when there was no supplementation with BMP-2 (cf. **Fig. 2A,C**). However, when BMP-2 was added to the AM, articular chondrocytes expanded in FGF-2 had higher GAG content per aggregate than did auricular chondrocytes at the same PD. The same was not true for chondrocytes expanded in both FGF-2 and IGF-1. In this instance, supplementation with IGF-1 plus FGF-2 seemed to decrease GAG expression

in articular chondrocytes but preserved GAG expression in auricular chondrocytes.

Histologic Assessment

To supplement the GAG data, histologic analysis of the microaggregates was performed, with Safranin O staining of GAGs and with Fast Green as a counterstain. **Figure 3** shows the results for auricular and articular chondrocytes, with and without BMP-2 addition, obtained from 1 of the 3 rabbits examined, whereas the results for the other 2 rabbits are shown in **Supplementary Figures S3** and **S4**. The histologic results parallel the GAG analysis data and show diminishing GAG, Safranin O staining, in aggregates from later passages. A stark contrast is easily discernable between articular chondrocytes grown in AM alone (**Fig. 3**, left side) and those with AM supplemented with BMP-2 (**Fig. 3**, right side), with the BMP-2-supplemented samples staining
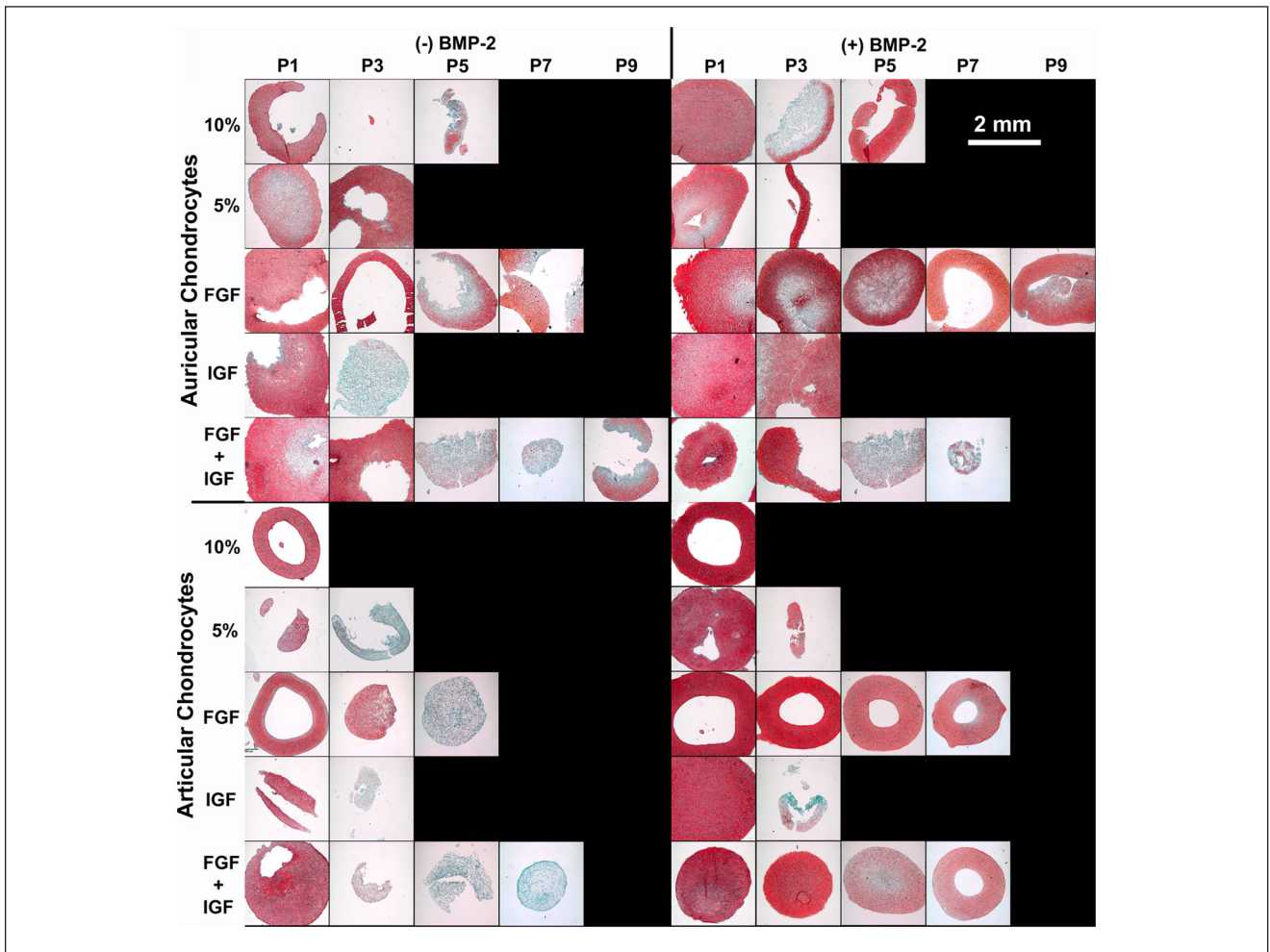
Figure 3. Histologic images of aggregate cultures of articular and auricular chondrocytes at passages (P) 1, 3, 5, 7, and 9 in either aggregate medium only ((–) BMP-2) or in aggregate medium plus BMP-2 ((+) BMP-2). Paraffin-embedded sections were stained with Safranin O to show glycosaminoglycans (GAGs) and counterstained with Fast Green. Blank spaces indicate that the cells had not grown sufficiently in expansion medium to perform the aggregate assay. BMP-2 = bone morphogenetic protein 2; IGF = insulin-like growth factor; FGF = fibroblast growth factor.

more intensely with Safranin O. A similar increase in Safranin O staining was observed in auricular chondrocytes too, although the magnitude of difference was not as high as that in articular chondrocytes, which is probably a result of the greater inherent baseline chondrogenesis of auricular chondrocytes compared to articular chondrocytes.

Discussion

The goal of this study was to test several growth factor combinations for their ability to support chondrogenic differentiation after culture expansion by promoting expansion without loss of chondrogenic potential, by enhancing chondrogenic differentiation directly, or both. The addition of FGF-2 under typical *in vitro* growth conditions is shown here to have a substantial potentiating effect on the life span and chondrogenicity of articular and auricular chondrocytes

alike. FGF-2 also appears to work synergistically with BMP-2 when added sequentially in the process from expansion to re-differentiation. The combined use of both FGF-2 during expansion and BMP-2 during differentiation effectively prolongs the chondrogenicity of both auricular and articular chondrocytes and allows chondrocytes to be expanded to approximately 20 PDs while retaining chondrogenic potential.

Several other studies have been reported on a variety of growth factors that have an impact on chondrocyte expansion and differentiation. Others have demonstrated the ability of FGF-2 to preserve the differentiation potential of human articular chondrocytes when added to expansion medium.[17,18] The study by Martin *et al.*[18] used bovine-derived articular chondrocytes, and their chondrogenic assays were conducted on cells cultured for only 2 passages. Another study using rabbit-derived articular chondrocytes showed that FGF-2

and IGF-1 in a defined medium were just as effective at expanding and differentiating rabbit articular chondrocytes as was serum-supplemented medium, but this study was restricted to expansion only to the end of passage 2.[15] The modest results that we observed with the addition of IGF-1 is similar to at least one study where rabbit auricular chondrocytes, when differentiated in serum-free medium containing IGF-1 and TGFβ2, contained more GAG per cell than serum-expanded chondrocytes, but this increase was only true for "young" (6-week-old) rabbit cells.[19]

For inducing chondrogenesis, BMP-2 has been shown to be chondrogenic in several cell types, including mesenchymal stem cells (MSCs),[20-22] embryonic stem cells,[23] and, most relevant to the present study, rabbit-derived articular chondrocytes.[10] In the study by Krawczak *et al.*,[10] BMP-2 was added to primary cultures of chondrocytes in serum-containing medium and chondrogenic stability was tested over time, and the results showed a negative result in that extensive exposure to BMP-2 produced diminished chondrogenic expression. In the study herein, BMP-2 was tested for its ability to promote chondrogenesis in serum-free aggregate cultures after cell expansion, so it is unclear whether long-term BMP-2 exposure in serum-free conditions would produce the same negative results as observed by Krawczak *et al.*, nor would we expect to need continuous BMP-2 exposure since these chondrocytes can express a chondrogenic phenotype even in the absence of BMP-2.

In many respects, our study of the expandability of chondrocytes regarding the retention of chondrogenic potential is a variation of the concept of "culture history" described by van Osch *et al.*,[24] where the culture context, such as surrounding matrix components, and expansion conditions can affect the ability of cells to re-differentiate. In this study, the growth factor "culture history" during expansion had a dramatic effect on the ability of those cells to differentiate later. One study was aimed at reversing detrimental culture history effects by reviving chondrogenic potential in late-passage "near-quiescent" rabbit articular chondrocytes by culturing them in a type II collagen matrix in medium supplemented with TGFβ.[25] The results showed a relative increase in aggrecan and type II collagen mRNA expression compared to controls, but there was no evidence of the formation of functional cartilage tissue from chondrocytes that had been "revived." The idea of including matrix signals in addition to growth factor signaling is promising, and future studies will be directed at the use of matrix carriers, such as collagen II[25] or synovial cell–derived matrix[26] in combination with growth factors for the expansion of chondrocytes for tissue engineering applications.

Although it is unlikely that these results will directly translate to the expansion and differentiation of human cells, the concept of finding conditions to preserve chondrogenic potential during expansion followed by enhancement of differentiation conditions is likely to apply to human cells as well. For example, FGF-2 has been shown to promote human chondrocyte expansion and later differentiation in both serum-containing[18] and serum-free medium,[27] and growth factors such as BMP-2[28] and growth differentiation factor-5 (GDF-5) plus IGF-1 have been shown to promote chondrogenesis in differentiation medium.[29] These and any number of other expansion and differentiation factors for human chondrocytes are likely to be even more effective when used in combination for both expansion and differentiation.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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