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Effects of passage number and post-expansion aggregate culture on tissue engineered, self-assembled neocartilage

Brian J. Huang [a], Jerry C. Hu [a], Kyriacos A. Athanasiou [a,b,*]

[a] Department of Biomedical Engineering, University of California Davis, USA
[b] Department of Orthopaedic Surgery, University of California Davis, USA

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ABSTRACT

Chondrocyte dedifferentiation presents a major barrier in engineering functional cartilage constructs. To mitigate the effects of dedifferentiation, this study employed a post-expansion aggregate culture step to enhance the chondrogenic phenotype of passaged articular chondrocytes (ACs) before their integration into self-assembled neocartilage constructs. The objective was twofold: (1) to explore how passage number (P2, P3, P4, P5, P6, and P7), with or without aggregate culture, affected construct properties; and (2) to determine the highest passage number that could form neocartilage with functional properties. Juvenile leporine ACs were passaged to P2–P7, with or without aggregate culture, and self-assembled into 5 mm discs in non-adhesive agarose molds without using any exogenous scaffolds. Construct biochemical and biomechanical properties were assessed. With aggregate culture, neocartilage constructs had significantly higher collagen content, higher tensile properties, and flatter morphologies. These beneficial effects were most obvious at higher passage numbers. Specifically, collagen content, Young's modulus, and instantaneous compressive modulus in the P7, aggregate group were 53%, 116%, and 178% higher than those in the P7, non-aggregate group. Most interestingly, these extensively passaged P7 ACs (expansion factor of 85,000), which are typically highly dedifferentiated, were able to form constructs with properties similar to or higher than those formed by lower passage number cells. This study not only demonstrated that post-expansion aggregate culture could significantly improve the properties of self-assembled neocartilage, but also that chondrocytes of exceedingly high passage numbers, expanded using the methods in this study, could be used in cartilage engineering applications.

Statement of Significance

This work demonstrated that extensively passaged chondrocytes (up to passage 7 (P7); expansion factor of 85,000) could potentially be used for cartilage tissue engineering applications. Specifically, an aggregate culture step, employed after cell expansion and before cell integration into a neocartilage construct, was shown to enhance the ability of the chondrocytes to form neocartilage with better biochemical and biomechanical properties. The beneficial effects of this aggregate culture step was especially noticeable at the high passage numbers. Most interestingly, P7 chondrocytes, which are typically highly dedifferentiated, were able to form neocartilage with properties similar to or higher than those formed by lower passage number cells. The ability to obtain high chondrocyte yields with an enhanced chondrogenic potential could have a broad, beneficial impact in improving current therapies (e.g., using higher cell seeding densities for repair) or developing new strategies that require high cell numbers, such as a scaffold-free approach in forming engineered cartilage.

1. Introduction

Tissue engineered cartilage has the potential to alleviate several shortcomings of current articular cartilage repair therapies. In a study examining 25,000 knee arthroscopies, 60% of knees had presence of articular lesions [1]. From a surgical perspective, an esti-

* Corresponding author at: Department of Biomedical Engineering, University of California Davis, One Shields Avenue, Davis, CA 95616, USA.
E-mail address: athanasiou@ucdavis.edu (K.A. Athanasiou).

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mated 250,000 articular cartilage repair procedures of the knee are performed annually in the U.S. [2]. Current cartilage repair therapies, however, do not consistently produce hyaline repair tissue, fill the entire defect, or integrate repair tissue with adjacent native tissue. Microfracture has been shown to form biomechanically inferior repair tissue, leading to its deterioration after 1.5–5 years [3–6]. Autologous chondrocyte implantation (ACI) and its matrix-assisted variants can result in inconsistent repair tissue; only 15–30% of treated defects were shown to develop hyaline-like repair tissue, while the rest developed a fibrocartilaginous fill [7–9]. These inconsistencies may arise from the placement of cells, which is surgeon-dependent, and maturation of the repair tissue, which is patient-dependent. Through a tissue engineering approach, functional neocartilage constructs can be consistently fabricated *in vitro* and used to replace damaged cartilage, potentially overcoming deficiencies of current therapies.

One method of engineering cartilage is using the self-assembling process to generate scaffold-free neocartilage [10]. Advantages of a scaffold-free approach include unobstructed matrix formation by scaffold-associated chemistry, complete biocompatibility, and potentially good integration due to the construct's high cellularity. Previously, self-assembled neocartilage had been generated using passaged articular chondrocytes (ACs) as a cell source. These constructs contained mostly type II collagen (little to no type I collagen) and had biomechanical properties close to juvenile native cartilage [11–13]. To form these constructs, ACs were first expanded under chondrogenically-tuned conditions, which involved the use of serum-free, FGF-2-supplemented medium and prolonged culture past cell confluence [11]. Cells were then cultured in aggregate suspensions to enhance redifferentiation of the dedifferentiated chondrocytes [12,14]. This aggregate culture step, central to the present study, will be discussed later. Finally, cells were dissociated and self-assembled in non-adherent agarose wells, where they secrete an abundance of cartilage-specific matrix and form a neocartilage construct [15]. In a previous study, optimization of the cell seeding density, at 2 million cells per 5 mm diameter disc, allowed the formation of homogeneous neotissues with hyaline-like matrix composition and tensile properties on par with native tissue values [13]. These scaffold-free neocartilage constructs can potentially be used to repair articular cartilage defects.

Because scaffold-free neocartilage constructs typically require high cell numbers for construct formation (e.g., 10 million cells/cm^2), expansion of chondrocytes to high passage numbers will be advantageous to overcome cell source limitations or to create large constructs. However, a caveat of chondrocyte expansion is the rapid loss of the chondrogenic phenotype after the first passage (P1) [16]. Chondrocyte dedifferentiation is marked by a progression from rounded to fibroblastic cell morphologies, an increase in cell size, and a decrease in secretion of cartilage-specific matrix [17,18]. Chondrogenic genes (e.g., SZP, COMP, aggrecan, collagen II, and SOX 9) are downregulated, while fibroblastic or mesenchymal genes (e.g., collagen I, collagen X, tenascin, and versican) are upregulated [16,19–23]. Although dedifferentiation is rapid, gene expression changes after each subsequent passage have been measured up to P6, indicating that progressive cellular changes still occur long after P1 [24]. Fortunately, chondrocyte redifferentiation can be induced by prolonged 3D culture (e.g., pellet culture, alginate encapsulation, suspension culture, culture within a scaffold, etc.). Chondrocytes expanded too extensively (approximately > P4), however, have been shown to lose their ability to partially or completely redifferentiate [19,25–28], rendering them unusable for cartilage engineering applications. Thus, chondrocyte dedifferentiation still poses a major barrier toward expanding chondrocytes to high passage numbers.

In a previous study, a post-expansion aggregate culture step was shown to enhance the ability of P4 ACs in forming self-assembled neocartilage with higher matrix content and biomechanical properties than neocartilage derived from cells that had not undergone the aggregate culture step [12]. Furthermore, these P4 AC-derived neocartilage constructs possessed equal or higher properties than constructs formed by lower passaged ACs, specifically, P0 and P3 ACs. These results suggest that ACs of even higher passage numbers (>P4), with aggregate culture, can potentially be used to form functional neocartilage. Employment of this aggregate culture step can potentially overcome the current notion that high-passage chondrocytes (>P4) are not suitable for use in cartilage engineering.

Allogeneic juvenile ACs were used in this study because this cell source has exhibited promising translational potential. Juvenile ACs have been shown to have significantly higher type II collagen gene expression [29,30] and drastically higher glycosaminoglycan (GAG) production than adult chondrocytes [29,31]. Juvenile ACs, after monolayer expansion, have also been shown to retain superior capabilities in secreting cartilage specific-matrix compared to expanded adult chondrocytes or stem cells [29]. Furthermore, an allogeneic source has been shown to be non-immunogenic [32] and require only one surgical procedure, as opposed to two for an autologous approach. Currently, allogeneic juvenile ACs are used in the FDA-approved product DeNovo® NT and the Phase III clinical product RevaFlex™ for cartilage repair. Therefore, allogeneic juvenile ACs are a cell source with high translational potential.

In this study, the effects of passage number (P2, P3, P4, P5, P6, and P7) – as modulated by a post-expansion, aggregate culture step – on the properties of self-assembled neocartilage were investigated in a full-factorial design. In addition to exploring the effects of passage number and aggregate culture on neocartilage properties, another objective was to determine the highest passage number that can be used to form constructs that still maintained functional biomechanical properties. At the end of 4 weeks of culture, constructs were evaluated for their gross morphology, matrix content, and biomechanical properties (compressive and tensile). Constructs formed with cells of higher passage numbers were hypothesized to exhibit diminished properties, as the cells become more dedifferentiated and less capable of synthesizing cartilage-specific matrix. The aggregate culture step was hypothesized to stimulate chondrocyte redifferentiation and rescue construct properties at the high passage numbers. The ability to create functional neocartilage with extensively passaged chondrocytes may be a boon toward reducing donor site morbidity, increasing feasibility to treat large articular lesions, and removing barriers for new treatments that might typically require high chondrocyte numbers.

2. Materials and methods

2.1. Chondrogenic medium formulation

The chondrogenic medium used throughout the study was composed of DMEM (25 mM glucose/GlutaMAX™; Life Technologies, Carlsbad, CA), 1% PSF (penicillin-streptomycin-fungizone; Lonza, Basel, Switzerland), 1% ITS (insulin-transferrin-selenium; BD Biosciences, San Jose, CA), 1% NEAA (non-essential amino acids; Life Technologies), 100 μg/mL sodium pyruvate (Thermo Fischer Scientific, Waltham, MA), 50 μg/mL ascorbate-2-phosphate (Sigma, St. Louis, MO), 40 μg/mL L-proline (Sigma), and 100 nM dexamethasone (Sigma).

2.2. Isolation of juvenile rabbit articular chondrocytes

ACs were isolated from full-thickness cartilage of the femoral condyle, trochlear groove, and tibial plateau of 6- to 8-week-old,