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Harnessing Biomechanics to Develop Cartilage Regeneration Strategies

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As this review was prepared specifically for the American Society of Mechanical Engineers H.R. Lissner Medal, it primarily discusses work toward cartilage regeneration performed in Dr. Kyriacos A. Athanasiou's laboratory over the past 25 years. The prevalence and severity of degeneration of articular cartilage, a tissue whose main function is largely biomechanical, have motivated the development of cartilage tissue engineering approaches informed by biomechanics. This article provides a review of important steps toward regeneration of articular cartilage with suitable biomechanical properties. As a first step, biomechanical and biochemical characterization studies at the tissue level were used to provide design criteria for engineering neotissues. Extending this work to the single cell and subcellular levels has helped to develop biochemical and mechanical stimuli for tissue engineering studies. This strong mechanobiological foundation guided studies on regenerating hyaline articular cartilage, the knee meniscus, and temporomandibular joint (TMJ) fibrocartilage. Initial tissue engineering efforts centered on developing biodegradable scaffolds for cartilage regeneration. After many years of studying scaffold-based cartilage engineering, scaffoldless approaches were developed to address deficiencies of scaffold-based systems, resulting in the self-assembling process. This process was further improved by employing exogenous stimuli, such as hydrostatic pressure, growth factors, and matrix-modifying and catabolic agents, both singly and in synergistic combination to enhance neocartilage functional properties. Due to the high cell needs for tissue engineering and the limited supply of native articular chondrocytes, costochondral cells are emerging as a suitable cell source. Looking forward, additional cell sources are investigated to render these technologies more translatable. For example, dermis isolated adult stem (DIAS) cells show potential as a source of chondrogenic cells. The challenging problem of enhanced integration of engineered cartilage with native cartilage is approached with both familiar and novel methods, such as lysyl oxidase (LOX). These diverse tissue engineering strategies all aim to build upon thorough biomechanical characterizations to produce functional neotissue that ultimately will help combat the pressing problem of cartilage degeneration. As our prior research is reviewed, we look to establish new pathways to comprehensively and effectively address the complex problems of musculoskeletal cartilage regeneration. [DOI: 10.1115/1.4028825]

Keywords: scaffoldless, cartilage, tissue engineering, biomechanics, meniscus, TMJ disc, integration, synergy

1 The Need for Functional Engineered Cartilage

Cartilage degeneration due to pathology or injury is one of the most significant problems of modern orthopaedics and creates a serious economic burden. For instance, arthritis and other rheumatic conditions cost over $320 billion in the United States alone in 2003 [1]. Osteoarthritis is particularly problematic within the elderly population, and it has been estimated that 10% of people over age 60 are affected [2]. Meniscectomy, which is used to treat meniscal tears, is the most common operation performed by orthopaedic surgeons in the United States [3]. Disorders of the TMJ are also quite common, as evidenced by the fact that 16–59% of the population have symptoms, and 33–86% exhibit clinical signs [4]. These afflictions diminish patient quality of life significantly as they hinder mobility, reduce independence, and make daily activities such as eating and talking painful. Unfortunately, consistently successful or widely acceptable cartilage replacement treatments do not exist, so physicians are typically limited to treating patients symptomatically [5] or to using cartilage repair methods that have limited clinical success [6]. The prevalence and severity of cartilage degeneration provides a strong impetus for engineering neocartilage-based treatment solutions.

The need for engineered cartilage has spurred the development of various tissue engineering technologies informed by biomechanical characterizations of native tissue and cells (Fig. 1). Cartilage has been extensively characterized at both the tissue and single cell levels, which has provided success criteria for functional cartilage tissue engineering. Initial studies with polymer scaffolds yielded exciting results for cartilage engineering, but numerous deficiencies still exist. Various scaffoldless approaches such as pellet culture, aggregate culture, and the self-assembling process have been developed to address these deficiencies. To further enhance these technologies, biochemical and biomechanical stimuli have been explored. Thus, informed by basic studies, cartilage tissue engineering has advanced toward translating in vitro work to clinical use.

2 Biomechanics as a Tissue Engineering Foundation

A wide range of biomechanical characterizations provided success criteria for tissue engineering studies and identified potential biochemical and biomechanical stimuli for in vitro cartilage

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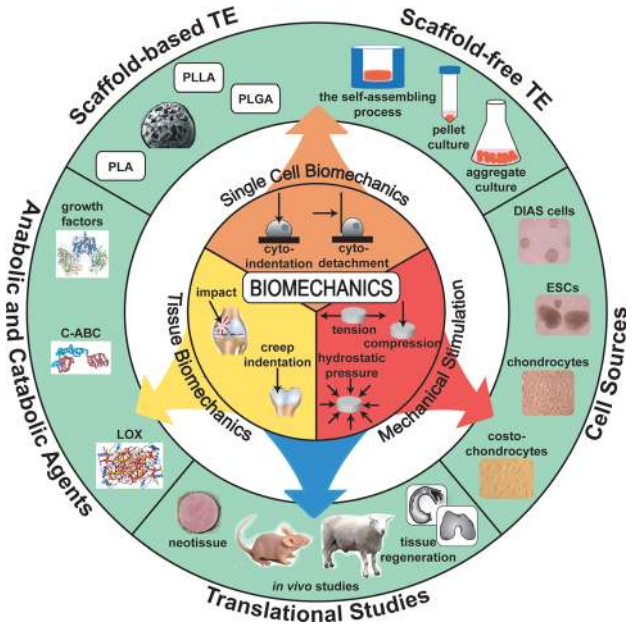

Fig. 1 The evolution of a comprehensive, multidisciplinary, and multiscale approach to elucidate cartilage physiology, pathology, and regeneration motivated by biomechanics

growth. Early studies investigated the biomechanics of normal and diseased articular cartilage, as well as the mechanics of various native tissues, which subsequently expanded to include single chondrocyte mechanics. Additionally, studies of the biomechanics of degeneration and impact-induced injuries have elucidated pathology mechanisms and potential therapies.

2.1 Biomechanical Properties of Tissues

2.1.1 Mechanical Properties Vary Topographically. Studies have shown variations of cartilage material properties. A creep indentation study of five species (bovine, canine, human, papio, and leporine) showed that the patellar groove exhibited the lowest aggregate modulus, lowest Poisson's ratio, and highest permeability [7]. Other studies have shown that the aggregate modulus of articular cartilage varies between 0.079 MPa and 2.1 MPa depending on depth [8]. Characterization of the compressive properties of other tissues also showed considerable topological variation. For example, the leporine meniscus had an aggregate modulus of 510 kPa in the anterior region and 120 kPa in the posterior region [9]. It was also found that in the bovine ankle joint, the tibial plafond exhibited threefold higher tensile properties and twofold higher compressive and shear moduli compared to its articulating talar dome. Neocartilage formed with cells isolated from these locations also exhibited these disparities [10]. Topographical examinations of mechanical properties of different tissues provide benchmark data for future tissue engineering studies of cartilage. Closely related tissues, for example, the opposing articulating surfaces of the ankle joint, exhibit differences in properties significant enough to warrant the consideration of the exact topographical source of cells or cartilage grafts.

In addition to assessing compressive mechanical properties, there has also been extensive characterization of cartilage tensile properties. For example, studies of the porcine TMJ disc showed great regional differences: the posterior region was found to be 2.5 times stiffer than the anterior region. The central and medial regions were 74% and 35% stiffer and 56% and 59% stronger than the lateral region, respectively [11]. Additionally, anisotropy was demonstrated in the fibrocartilaginous TMJ discal attachments. Anteroposteriorly, the lateral attachment was stiffest (8.3 MPa) compared to the anterior superior (1.4 MPa) attachment [12]. Mediolaterally, the posterior superior attachment stiffness (16.3 MPa) exceeded that of the medial attachment (1.4 MPa) [12]. Tensile properties of articular cartilage also exhibit substantial depth dependence, decreasing by 40% from the superficial zone to the deep zone [13].

2.1.2 Tissue Type Significantly Influences Mechanical Properties. In addition to topological variation, biomechanical properties depend heavily on tissue type and species. For instance, the TMJ disc was found to be 15–60 times softer in compression than hyaline cartilage from the hip and knee joints [14]. Creep indentation of meniscus tissue showed striking differences among species: the aggregate modulus of canine and porcine models was 150 kPa and 270 kPa, respectively [9]. Another study demonstrated that aggregate modulus and shear modulus of human meniscus were most similar to the bovine model, but the human meniscus permeability was closest to canine and baboon values [15]. These studies provided valuable information for selecting appropriate animal models.

2.1.3 Pathology Influences Biomechanical Properties. Various pathologies have been shown to alter the properties of cartilage, which could help explain risk factors for musculoskeletal injuries. Sheep with depleted estrogen were shown to display lower aggregate moduli in knee articular cartilage [16]. By elucidating the relationship between cartilage integrity and estrogen, these characterizations might elucidate the pathogenesis of osteoarthritis in postmenopausal women. Additionally, this model could be used to investigate potential therapies that reduce the biomechanical symptoms of menopause. Biomechanical studies also showed that cartilage is substantially softer in diabetic patients [17]. For example, cartilage from patients with diabetes had a 38% lower aggregate modulus, 37% lower shear modulus, and 111% higher permeability values compared to nondiabetic patients.

In the TMJ, the greatest strains were observed in the lateral attachments in the mediolateral direction and the posterior superior attachment in the anteroposterior direction [12]. These correspond to the most common directions of disc displacement, suggesting compromised attachments contribute to disc displacement [12].

2.2 Single Cell Biomechanics. In addition to tissue-level biomechanical studies, mechanics have been investigated at the cellular level. Quantifying single cell mechanics has elucidated the underlying mechanisms that govern chondrocyte mechanobiology. By modeling the biological responses to cellular deformation, a deeper understanding of chondrocyte responses to loading and mechanotransduction at the single cell level have been developed (Fig. 2). A thorough knowledge of mechanotransduction at the single cell level can inform the selection of appropriate stimuli for tissue engineering.

2.2.1 Cytodetachment Quantifies Cell Adherence. Cytodetachment methods were developed to quantify the force required to displace attached cells. It has been shown that cellular adhesion plays an important role in embryonic development, which is significant for tissue engineering applications that often attempt to recapitulate development. When articular chondrocytes were cultured on various substrates, substrate-dependent adhesive forces were demonstrated [18]. By quantifying the force required to displace the chondrocytes from each substrate, it was shown how cells adhere differently to different materials. This work has widespread implications because many tissue engineering techniques involve culturing cells on substrate materials.

2.2.2 Cytocompression Alters Gene Expression and Deforms Nuclei. Compressing chondrocytes has been shown to influence gene expression, potentially due to nuclear deformation. For instance, statically compressing chondrocytes was found to modulate gene expression of extracellular matrix (ECM) proteins in a

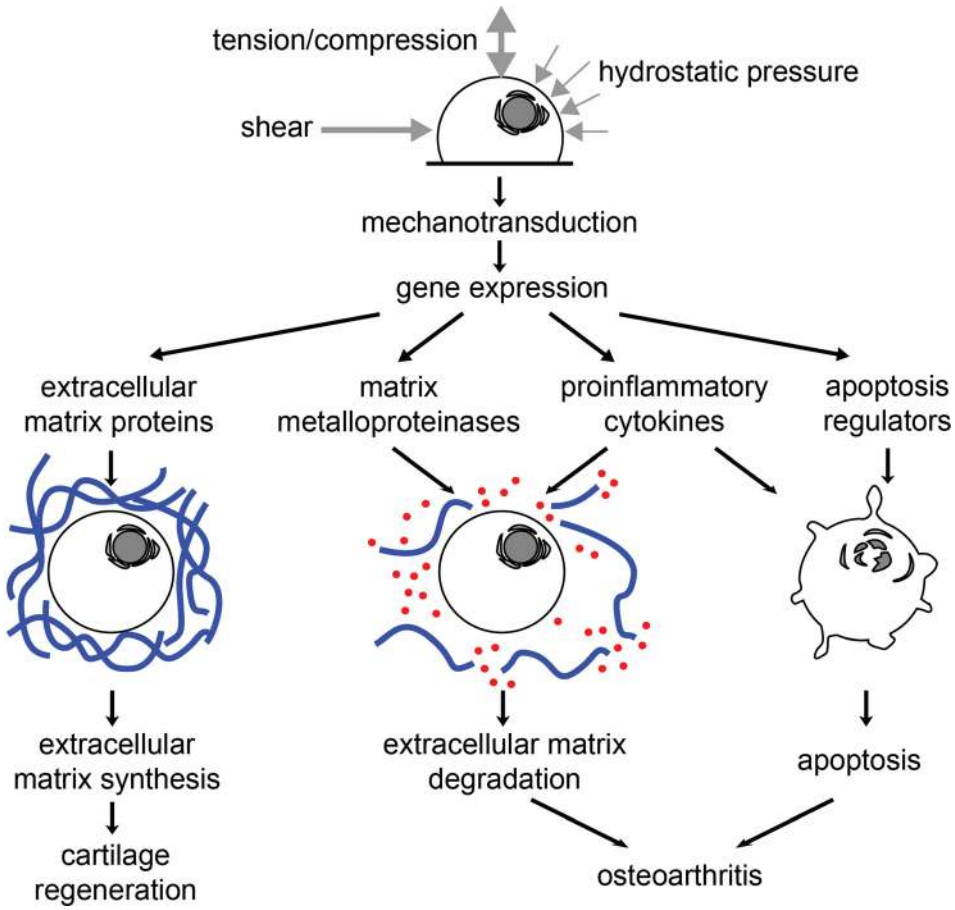

Fig. 2 The single chondrocyte approach to elucidate mechanotransduction pathways and to select biomechanical forces as exogenous stimuli for tissue engineering strategies

dose-dependent manner [19]. Increased force exposure catabolically shifted single cell mRNA levels of aggrecan, collagen type II, and tissue inhibitor of metalloproteinase-1. This work showed that single cells respond to static compressive force by modifying gene expression related to ECM synthesis and maintenance.

Cytocompression studies also showed that when the entire chondrocyte was compressed, cell to nuclear strain was 1:1. This was an unexpected result as isolated nuclei have been previously shown to be substantially stiffer than the rest of the cell. The significant in situ nuclear deformation seen with cytocompression shows that mechanotransduction proceeds, at least partly, through a pathway via direct conformational changes of chromatin (Fig. 3) [19].

2.2.3 Growth Factors Stiffen Cells and Mitigate Effects of Compression. Growth factor application has been shown to influence chondrocytes at both the gene and cellular level. Single cell real-time polymerase chain reaction (RT-PCR) showed that cells treated with transforming growth factor beta-1 (TGF-β1) and insulin-like growth factor-I (IGF-I) recovered aggrecan and metalloproteinase-1 gene expression following static compression, suggesting that growth factors can reduce the detrimental effects of abnormal forces [19] (Fig. 4). Growth factor treatment also increased F-actin levels in chondrocytes, which could contribute to the observed stiffening [20]. Furthermore, creep cytoindentation studies showed that IGF-I and TGF-β1 treatment increased the stiffness coefficient of single chondrocytes approximately twofold [21]. These results suggest that growth factor application influences cellular mechanics and also may provide mechanoprotection.

2.3 Cartilage Impact Mechanics

2.3.1 Impact results in Delayed Cartilage Degeneration. Because many cartilage pathologies result from trauma, the effect

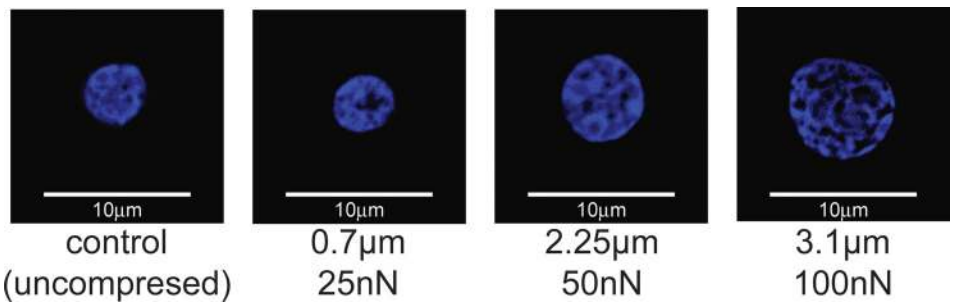

Fig. 3 Nucleus images of chondrocytes compressed at 0, 25, 50, and 100 nN (left to right). Transcriptional changes may be a direct result of conformational changes of chromatin. (Figure adapted from Leipzig and Athanasiou [19]).

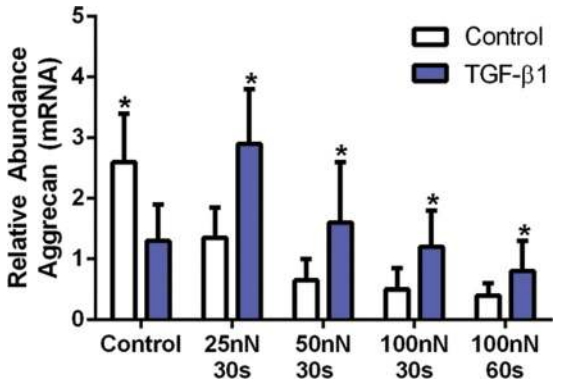

Fig. 4 Aggrecan gene expression levels for single chondrocytes subjected to various static compression conditions. There is a dose-dependent catabolic response of single cells to the applied static force. The growth factor, however, seems to offer a mechanoprotective effect. (Figure adapted from Leipzig and Athanasiou [19]).

of impact-induced injuries has been studied. In addition to the intuitive result that critical impact levels induced cell death, it has been shown that even subcritical level impacts create a degenerative response. For example, by 24 hours following impact, cell death increased and tissue stiffness decreased [22]. These degradative changes persisted at 1 week and were further accompanied by measurable changes in ECM biochemistry. Additionally, at 4 weeks following impact, cell death increased and tissue stiffness decreased [23]. These studies identified possible time windows and success criteria to be used in future studies employing intervention agents.

2.3.2 Exogenous Agents Mitigate Impact Injury. To ameliorate degenerative changes due to impact, several chemical agents were examined as postinjury treatments. It was shown that, for example, applying 100 mm doxycycline following impact reduced cumulative glycosaminoglycan (GAG) release at 1 and 2 weeks by 30% and 38%, respectively [24]. Similarly, IGF-I administration decreased GAG release by 49%, while applying the nonionic surfactant P188 following impact reduced cell death by 75% [25]. These studies demonstrated several potential methods for mitigating detrimental effects of impact injury.

3 Scaffolds for Cartilage Repair

Many studies have examined the use of poly(glycolic acid)-poly(lactic acid) (PLA-PGA or PLGA) copolymers as scaffolds for cartilage tissue engineering and repair. Examining the in vitro properties of these copolymers showed how they could be used for tissue regeneration. Additionally, in vivo studies demonstrated that PLGA can be employed for articular cartilage repair.

3.1 Biodegradable Scaffolds

3.1.1 In Vitro Studies Elucidate PLGA Degradation. Early tissue engineering work focused on understanding relevant in vitro characteristics of PLGA scaffolds. For example, studying release kinetics of a trypsin inhibitor from a PLGA implant showed sigmoidal kinetics with increased release between 3 and 7 weeks [26]. Results also demonstrated that cyclic compressive loading significantly slowed the decrease of molecular chain size during the first week, significantly increased protein release for the first 2–3 weeks, and significantly stiffened the implant for the first 3 weeks. In addition, this study showed that dynamic loading and the environment in which an implant was placed affect its biodegradation [27]. By providing a greater understanding of the degradation properties of PLGA, these studies provided a foundation for future in vivo studies.

One deficiency of biomaterials, such as PLGA, has been their propensity to undergo bulk degradation with concomitant sudden decreases in *p*H. Various material modifications such as incorporating basic salts have been investigated to mitigate detrimental *p*H changes during PLGA degradation. For example, PLGA implants containing calcium carbonate maintained *p*H values above 7.4, whereas the *p*H of control scaffolds dropped to 3.0 [28]. The technique of basic salt incorporation is now widely used by manufacturers of biodegradable screws and other fixation devices.

3.1.2 In Vivo Studies Illustrate Biocompatibility and Tissue Engineering Potential. In Vivo studies have shown the biocompatibility of PLGA in various animal models. PLGA has been employed to repair full-thickness osteochondral defects in goat biphasic implants, with each phase exhibiting bone-like and cartilage-like properties, respectively [29]. After 16 weeks of treatment, substantial tissue regeneration was observed, and the implant integrated with the subchondral bone. Studies in rabbits showed that poly(L-lactic acid) (PLLA) scaffolds were biocompatible and promoted the formation of cartilaginous repair tissue that had similar morphological, histological, and biochemical properties to native tissue [30,31]. These studies, among others, have highlighted the potential of using PLGA scaffolds to regenerate functional cartilage.

Combinations of different polymers have been employed to capitalize on the advantages of each component of the scaffold. For example, a PLA/alginate scaffold was used to promote chondrogenic differentiation of MSCs [32]. More recent fabrication technologies have also enabled the synthesis of anisotropic composite scaffolds that could more closely mimic the anisotropy of native tissue [33]. By leveraging the benefits of multiple materials, composite scaffolds have vastly expanded the breadth of scaffold-based tissue engineering.

3.2 Enhancing Scaffold-Based Tissue Engineering

3.2.1 Growth Factors Enhance Matrix Production. Growth factors such as IGF-I and the TGF-β superfamily, which includes forms of TGF-β and the bone morphogenetic proteins (BMPs), have been extensively investigated for their beneficial effects on tissue neocartilage. For instance, administering TGF-β increased GAG deposition in three-dimensional cultures of equine chondrocytes [34], rabbit auricular chondrocytes [35], and bovine articular chondrocytes [36]. Other studies have shown that BMP-2 increases GAG deposition in explant cultures [37] and in engineered cartilage [38]. Many BMPs have also been shown to promote collagen synthesis. For example, BMP-2 [39] and BMP-7 [40] have been employed to increase collagen deposition. IGF-I treatment has been shown to increase GAG production in both explants [41] and engineered neocartilage [42,43]. For bovine articular chondrocytes cultured on alginate beads, IGF-I increased collagen gene expression and deposition [44], but it did not impact the number of collagen crosslinks [45]. These studies identified multiple anabolic agents that can be applied to induce matrix production.

3.2.2 Mechanical Stimuli Enhance Mechanical Properties and Matrix Production. Direct compression has been used to modulate matrix composition and concomitantly influence neocartilage properties. Direct compression at various frequencies and strain levels has been shown to increase collagen deposition [46,47]. Additionally, dynamic loading of cell-seeded agarose scaffolds increased the equilibrium aggregate modulus sixfold and also significantly increased the GAG and collagen content [46]. The ability of direct compression to modulate biochemical and biomechanical properties showed its importance as stimulus for cartilage engineering.

Hydrostatic pressure also acts as a potent mechanical stimulus for in vitro cartilage growth. For example, studies on PGA meshes have shown that hydrostatic pressure can increase matrix

production [48,49]. Despite these promising results, hydrostatic pressure has also been shown to be deleterious. In particular, applying hydrostatic pressure above physiological levels exhibited harmful effects and led to decreased matrix production and increased expression of inflammatory signaling cytokines such as interleukin-6 and tumor necrosis factor [50]. These studies showed the potential of hydrostatic pressure to improve neocartilage properties by choosing an appropriate stimulation regimen.

3.2.3 Combining Mechanical Stimuli and Growth Factors Synergistically Increases Compressive Properties. Since PGA was noted to degrade too fast, scaffold work was extended to PLLA, which is known to degrade more slowly. TGF-β1 was identified as a potent bioactive agent acting on fibrochondrocyte-seeded PLLA; it increased collagen and GAG deposition 15-fold and eightfold, respectively. When this growth factor treatment was combined with hydrostatic pressure it resulted in additive increases in collagen and GAG deposition, and synergistic increases in compressive properties [51]. Similarly, dynamic loading of chondrocyte-seeded agarose hydrogels treated with TGF-β1 or IGF-I synergistically increased the aggregate modulus of 277% or 245%, respectively.

4 Beyond Scaffolds: New Tissue Engineering Strategies

Scaffoldless tissue engineering employs high density cell culture to create cartilage neotissue. These tissue engineering approaches have several advantages over scaffold-based strategies including increased retention of phenotype, increased cell–cell contact, and lack of degradation products. Although many scaffoldless culture methods exist, most of the techniques depend on high density culture to foster cell–cell interactions. Three commonly used approaches include pellet culture, aggregate culture, and the self-assembling process.

4.1 Scaffoldless Approaches

4.1.1 Pellet Culture Reproduces Cartilage Phenotype. One of the most common scaffoldless methods is pellet culture, which entails the forced aggregation of cells via centrifugation to create a high density pellet. Pellet culture has been applied to a wide range of cells including growth plate chondrocytes [52] and hyaline chondrocytes [53]. This culture method has been used to produce collagen networks that have the same composition and fibril sizes as explant cultures [54]. Additionally, pellet culture has been employed to induce cartilage differentiation of human adult stem cells [55]. Pellet culture has shown promising results regarding chondrocyte phenotype, but the restricted sizes and shapes of the neocartilage have limited the translatability of pellet cultures.

4.1.2 Aggregate Culture Promotes Chondrogenic Development. Aggregate culture was developed by suspending chondrocytes in solution in a rotating, nonadherent culture environment which enabling them to aggregate and thereby increases the cell–cell interactions that have been shown to maintain the chondrocytic phenotype [56]. This culture method has been employed to demonstrate the chondrogenic potential of mesenchymal stem cells [57–59] and synovial cells [60]. Aggregate culture has also been used for cartilage tissue engineering applications. For instance, aggregate culture promoted gene expression of collagen type II and aggrecan without collagen type I expression [53]. These studies illustrated the ability of aggregate culture to promote phenotype maintenance and chondrogenic differentiation. To that end, aggregate culture has been used to redifferentiate and restore chondrocytic phenotype of articular chondrocytes, fibrochondrocytes, and costochondral cells after monolayer expansion. Aggregate culture increased GAG/wet weight (WW), collagen type II:collagen type I ratio, and compressive properties of neocartilage using articular chondrocyte and fibrochondrocytes [61].

4.1.3 The Self-Assembling Process Recapitulates Cartilage Development. When chondrocytes were cultured at high density in nonadherent molds without exogenous aggregation forces, the cells coalesced to form neocartilage that not only appeared to be hyaline cartilage-like but also had functional properties of the same order of magnitude as native values [62]. The self-assembling process was developed based on the differential adhesion hypothesis to produce robust neocartilage (Fig. 5) [62] and is a unique tissue engineering technique that mimics many aspects of native cartilage development [63]. For example, on a dry weight (DW) basis, these engineered neocartilage contained 2/3 more GAG than native tissue. Collagen reached 1/3 the level of native tissue, and the compressive stiffness reached more than 1/3 of native tissue values. This progress toward achieving native biomechanical properties and matrix composition was exciting as it provided early validation for this scaffoldless approach.

These promising results spurred an investigation of the mechanism underlying the self-assembling process (Fig. 6) [64]. Increased N-cadherin expression during neotissue formation suggested that the process is mediated by differential adhesion. Also, several biochemical properties recapitulated cartilage development including an increased proportion of collagen type II, decreased proportion of collagen type VI, decreased chondroitin 6- to 4- sulfate ratio, and localization of collagen VI to the pericellular matrix. In addition, the compressive properties reached a plateau and tensile characteristics peaked at 4 weeks. These studies showed that the self-assembling process mimicked tissue development and maturation, suggesting that a set of exogenous stimuli could be applied to augment tissue functional properties.

4.2 Mechanical Stimuli

4.2.1 Hydrostatic Pressure Enhances Mechanical Properties. Hydrostatic pressure stimulation was shown to be advantageous during the self-assembling process. After applying hydrostatic pressure of different magnitudes, durations, and frequencies, a particularly effective regimen was identified. Applying 10 MPa static hydrostatic pressure significantly increased the aggregate modulus by 1.4-fold. It was exciting to note that this regimen also affected functional properties that seem to be difficult to improve upon, namely, tensile modulus and strength along with corresponding collagen content, which increased over twofold [65]. For the first time, this study examined the immediate and long-term effects of hydrostatic pressure on biomechanical properties, and demonstrated that hydrostatic pressure has an optimal application time in neocartilage development.

4.2.2 Direct Compression Increases Compressive and Tensile Properties. As hydrostatic pressure was proven to be such a potent stimulator, it became apparent that other biomechanical stimuli should to be examined. Applying dynamic compression to medial meniscal explants showed an up-regulation of aggrecan expression by 108% [66]. The beneficial effects of dynamic compression were also observed in self-assembling neocartilages, where applying 17%, 0.1 Hz compression, for example, was found to increase the aggregate modulus by 70%. These results have

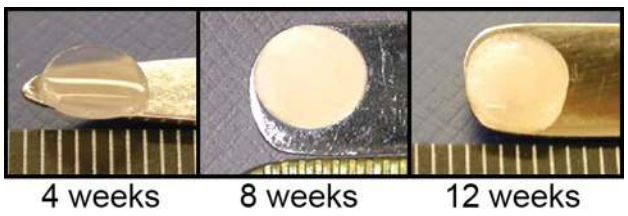

Fig. 5 The self-assembling process results in cartilage with clinically relevant dimensions. (Figure adapted from Hu and Athanasiou [62]).

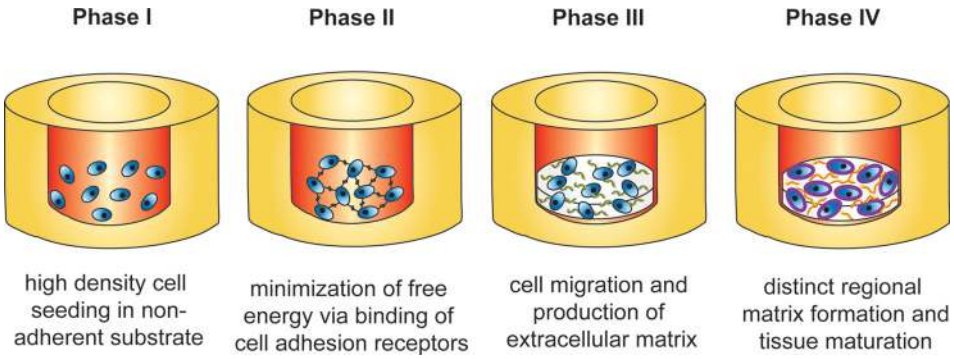

Fig. 6 In the first phase of the self-assembling process, chondrocytes were seeded at high density in a nonadherent agarose mold. In the second phase, cells began to aggregate following the differential adhesion hypothesis, which states that maximized intercellular adhesion occurs when the total free energy of the forming neocartilage is minimized. Tissue formation occurs during the self-assembling process via only cell–cell interactions, whereas in scaffold-based approaches it is achieved via cell-scaffold interactions. In the third phase, neotissue begins to form, and cells migrate apart and secrete ECM. In the fourth phase, neocartilage exhibits significant maturation, including distinct pericellular ECM formation and localization of collagen type VI. (Figure adapted from Athanasiou et al. [63]).

shown the potential of direct compression to further improve engineered neocartilage.

Direct compression of meniscus-shaped neocartilage composed of chondrocytes and fibrochondrocytes stimulated the neotissue in both tension and compression due to the wedge shape of the tissue. This mechanical stimulation increased the compressive relaxation modulus up to 66%, compressive instantaneous modulus up to 54%, circumferential tensile modulus up to 65%, and radial tensile modulus up to 200% [67]. This study advanced the field of meniscal tissue engineering by improving all major functional properties of scaffoldless meniscus neocartilage following tension-compression stimulation.

4.3 Exogenous Agents

4.3.1 Ion Channel Modulation may Supplement Mechanical Stimuli. The effects of hydrostatic pressure are known to be mediated by ion channels [68]. Motivated by the mechanism of action of hydrostatic pressure, inhibitors of Na^+ ion transporters and stimulators of intracellular Ca^{2+} were investigated as possible actors in the development of self-assembling neocartilage [69]. Applying ouabain (Na^+/K^+-ATPase inhibitor), bumetanide ($Na^+/K^+/2Cl^-$tritransporter inhibitor), histamine (cAMP activator), and ionomycin (a Ca^{2+} ionophore) to self-assembling neocartilage for 1 h daily on days 10–14 of culture showed that 20 μM ouabain, 0.3 μM ionomycin, or their combination increased the tensile modulus by 40–95% (Fig. 7). Furthermore, the 20 μM ouabain treatment increased the ultimate tensile strength by 56–86% at 4 weeks [69]. This study was the first to show that altering intracellular ion concentrations can increase the mechanical properties of engineered articular cartilage. In addition, these results have important relationships to hydrostatic pressure mechanotransduction.

4.3.2 Growth Factors Increase Compressive and Tensile Properties. Various growth factors, applied individually and in combination, were investigated to improve the functionality of self-assembling neocartilage. For example, a combination treatment of BMP-2 and IGF-I double the aggregate modulus, accompanied by increases in GAG production. However, TGF-β1 was found to be the most potent growth factor, inducing doubling of both aggregate modulus and tensile modulus, and increasing GAG and collagen content [70]. Additionally, continuous treatment of chondrocyte and fibrochondrocyte cocultures with TGF-β1 resulted in functional properties within the range of native TMJ disc values and increased collagen deposition by 20%, compressive stiffness by 130%, and tensile modulus by 170% relative to untreated controls [71]. These findings are exciting as coupling growth factor application with the self-assembling process

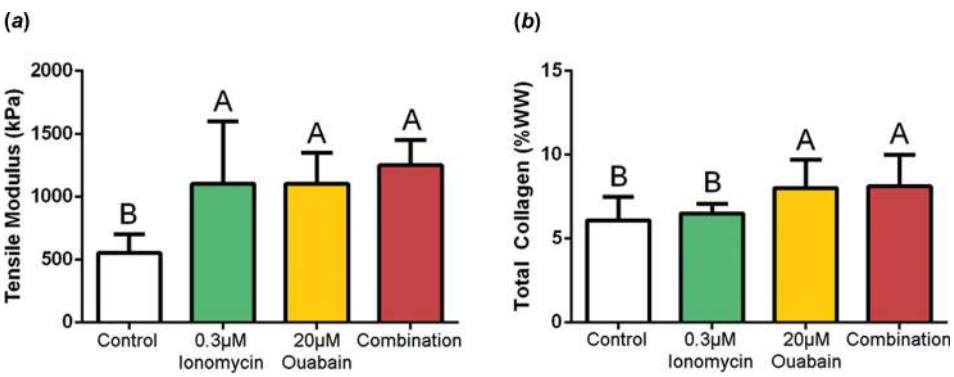

Fig. 7 Tensile stiffness (A) and total collagen normalized to neocartilage WW (B). All three treatments resulted in an ~95% increase in tensile stiffness compared with control, while groups treated with ouabain contained significantly more total collagen per wet weight than controls. Bars show the mean and SD. *= $p < 0.05$. (Figure adapted from Natoli et al. [69]).

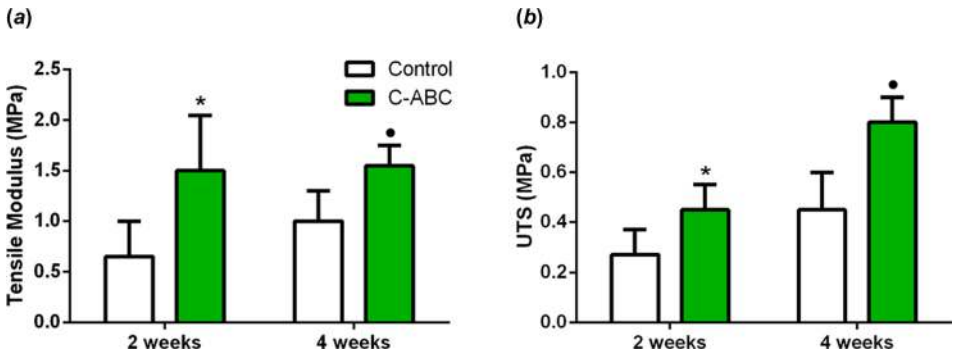

Fig. 8 C-ABC treatment increased both the tensile modulus and tensile strength of self-assembling neocartilage. (Figure adapted from Natoli et al. [73]).

resulted in engineered neocartilage with substantially improved functional properties.

4.3.3 C-ABC Increases Tensile Properties of Native Tissue and Engineered Neocartilage. The enzyme Chondroitinase-ABC (C-ABC) has been applied to deplete GAG content and subsequently improve biomechanical properties of both native tissue and engineered neocartilage. For example, applying C-ABC to bovine articular cartilage explants decreased GAG and increased tensile integrity [72]. C-ABC also increased tensile properties of self-assembling articular cartilage (Fig. 8) without compromising compressive properties, as GAG levels return post-treatment [73]. Multiple C-ABC treatments of self-assembled neocartilage further increased tensile properties, reaching values of 3.4 and 1.4 MPa for the tensile modulus and ultimate tensile strength, respectively [74]. C-ABC represents an exciting method for engineering functional articular cartilage by departing from conventional anabolic approaches.

4.3.4 LOX Increases Collagen Crosslinks and Neocartilage Tensile Properties. LOX is an enzyme responsible for the formation of pyridinoline crosslinks of collagen. It has been shown that hypoxia increases LOX gene expression via HIF-1α and therefore promotes collagen crosslinking [75]. Notably, the timing of hypoxia application elicited distinct responses. When applied during the third and fourth week of neocartilage culture, hypoxia increased the amount of pyridinoline 34% over controls, increased LOX gene expression 18-fold, and increased tensile stiffness 80% over controls. However, hypoxia application at earlier time points did not result in improved properties, indicating LOX increases neocartilage tensile properties when acting upon mature collagen, not during collagen synthesis [75]. Additionally, adding exogenous LOX, copper sulfate, and hydroxylysine to neocartilage cultures resulted in a synergistic increase of pyridinoline crosslinks tenfold over controls, manifesting in a 3.3-fold increase in tensile properties [76]. Thus, exogenous LOX is a potent mediator of collagen crosslinking, possibly removing the need for hypoxic culture.

4.4 Synergisms Among Stimuli. Natively, developing cartilage experiences an array of both mechanical and biochemical stimulation simultaneously [77]. Through in vitro application and analysis, it has been found that many of these stimuli act synergistically to enhance the functional properties of neocartilage.

4.4.1 Hydrostatic Pressure and TGF-β1 Synergistically Increase Neocartilage Collagen Content. After identifying individual potent biochemical and biomechanical stimuli, the next logical step was to combine the optimal regimens of each stimulus to further improve the properties of the engineered neocartilage. For example, combining 10 MPa static hydrostatic pressure treatment with 30 ng/ml TGF-β1 administration had an additive effect on the mechanical properties of neocartilage, increasing the aggregate modulus by 164% and the tensile modulus by 231%, approaching 300 kPa and 2 MPa, respectively (Fig. 9). Additionally, the combined treatment had a synergistic effect on collagen content, increasing it by 173% [78].

4.4.2 C-ABC and TGF-β1 Synergistically Enhance Neocartilage Collagen Content and Mechanical Properties. Chondrocyte neocartilage treated with C-ABC and TGF-β1 exhibited in vitro maturation, attaining biochemical and biomechanical values approaching native values. Treatment synergistically increased neocartilage collagen content. This study also proposed that TGF-β1 increased collagen biosynthesis via increased MAPK signaling and that C-ABC promotes maturation of the collagen network by a biophysical rather than a genetic mechanism [79]. The two

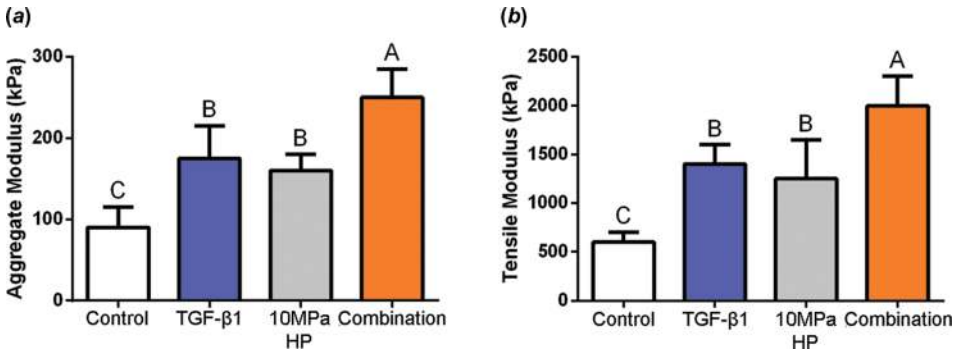

Fig. 9 Combination of hydrostatic pressure stimulation and growth factors increased compressive (*a*) and tensile (*b*) properties of tissue-engineered cartilage. (Figure adapted from Elder and Athanasiou [78]).

distinct mechanisms underlying this treatment help to explain the synergistic effects of combining these stimuli.

In a coculture of chondrocytes and fibrochondrocytes, C-ABC and TGF-β1 treatment synergistically increased meniscus-shaped neocartilage radial tensile modulus fivefold and compressive relaxation modulus by 68% [80]. This study showed that tissue engineering can produce meniscus neotissue with functional properties on par with native tissue. Toward TMJ disc tissue engineering, C-ABC and TGF-β1 treatment synergistically increased tensile modulus and ultimate tensile strength of neocartilage composed of chondrocytes and fibrochondrocytes. This study also elucidated the possible biophysical mechanism of C-ABC action. C-ABC temporarily depletes GAG, which allows for the formation of a more functional collagen network and a GAG:collagen ratio close to native values [81].

4.4.3 C-ABC, TGF-β1, and LOX Synergistically Increase Neocartilage Tensile Properties. Further combining mediators of neocartilage functional properties, C-ABC, TGF-β1, and LOX treatment of neocartilage composed of chondrocytes and fibrochondrocytes synergistically increased both tensile modulus (202%) and ultimate tensile strength (121%) at 6 weeks of culture. These enhancements persisted at 12 weeks of culture. Collagen fibril diameter was also synergistically increased by 104% at 12 weeks [82].

4.5 Engineering Other Tissue Types. Based on the promising results of engineering using articular chondrocytes, tissue engineering was expanded to other types of cartilage. A spectrum of cartilaginous tissues has been created by modulating the cell source and biochemical stimuli. Additionally, numerous novel molds have been developed to self-assemble a wide variety of tissue geometries. These approaches have enabled the engineering of various fibrocartilage tissues, such as the meniscus and TMJ.

4.5.1 The Self-Assembling Process can be Customized to Specific Tissue Geometries. In addition to using different cell sources to create a spectrum of cartilages, different molds were examined to engineer native tissue geometries and anisotropy. For instance, an annulus-shaped mold was used to produce ring-shaped meniscus neocartilage. Neocartilage grown in meniscus molds resulted in circumferential alignment of collagen fibrils, which was particularly exciting considering the difficulty of recapitulating the collagen network in vitro. This anisotropic collagen orientation created a threefold increase in circumferential tensile properties compared to radial tensile properties (Fig. 10) [83]. The ability to control both the shape and the collagen orientation in the neocartilage represents a major step forward in engineering fibrocartilaginous tissues.

The self-assembling process has also been used to engineer tissues with zonal variation. For example, anisotropic and zonally variant meniscus neocartilage was produced by self-assembling the inner meniscus (100% chondrocytes) followed by cell seeding the outer meniscus (coculture of chondrocytes and fibrochondrocytes). After 4 weeks, the inner and outer zones exhibited different GAG/DW, 42% and 62%, respectively. In contrast, the circumferential tensile modulus and collagen/DW of the outer zone was 101% and 129%, respectively, higher than that of the inner zone. There was no difference in the radial tensile modulus between the zonally variant engineered meniscus neocartilage and neocartilage composed completely of a coculture of chondrocytes and fibrochondrocytes, suggesting the inner and outer zones of the zonally variant neocartilage integrated [84].

4.5.2 Culture Conditions can be Altered to Create a Spectrum of Tissues. Modulating the cell type and applied biochemical stimuli allowed the engineering of various fibrocartilages [85]. In particular, coculturing articular chondrocytes and fibrochondrocytes enabled the engineering of a range of cartilaginous tissues. Altering the presence of serum or growth factors further controlled neotissue properties. For example, by varying these conditions, it was possible to manipulate the collagen type II:collagen type I ratio. Neocartilage also exhibited many enhanced mechanical properties, including a compressive stiffness of 128 kPa and a tensile modulus of 3 MPa. These results suggested that cocultures and biochemical stimuli could be coupled with the self-assembling process to engineer fibrocartilages such as the meniscus.

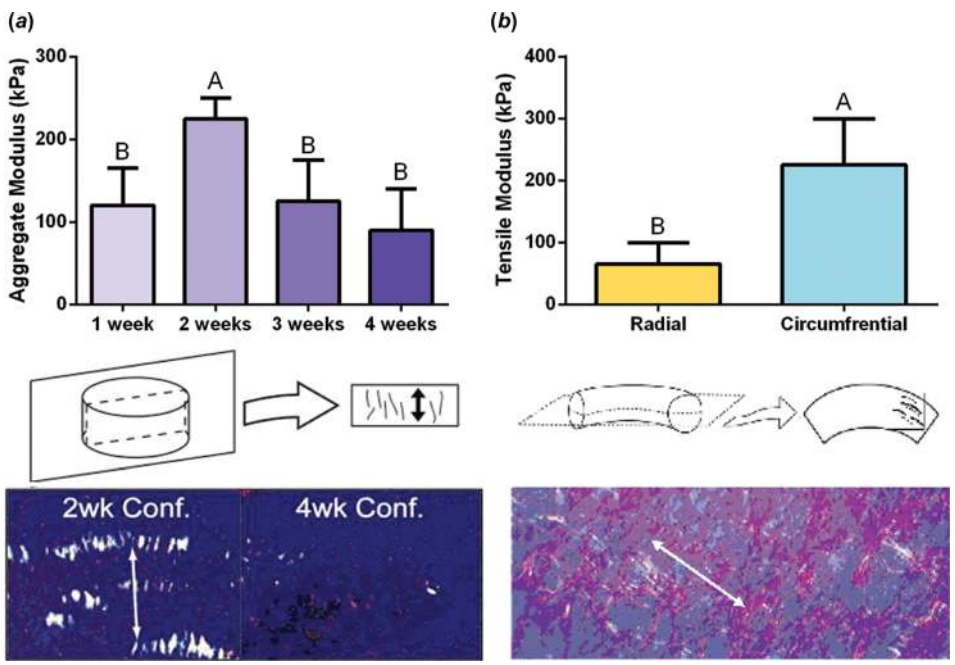

Fig. 10 Collagen alignment, as confirmed by polarized microscopy, contributes to (*a*) a higher aggregate modulus ($p < 0.05$) for articular neocartilage confined for 2 weeks; and (*b*) a threefold increase in circumferential, compared to radial, tensile modulus in meniscus neocartilage. (Figure adapted from Aufderheide and Athanasiou [83]).

In terms of TMJ disc tissue engineering, initial studies indicated that a combination of L-glutamine, sodium pyruvate, and insulin improved cell proliferation rates without affecting collagen production or gene expression [86]. The self-assembling process was then applied to TMJ disc cells [87]. TMJ disc cells were noted to contract significantly, so costal chondrocytes were used instead. Costal chondrocyte neocartilage produced almost 40 times more collagen and 800 times more GAG than TMJ disc cell neocartilage. This study demonstrated the ability of costal chondrocytes to produce ECM that may function in a TMJ disc replacement. Additionally, varying the duration of aggregate culture of expanded costochondral cells generated a spectrum of fibrous to hyaline neocartilage with corresponding ratios of collagen type II:collagen type I [88]. Therefore, aggregate culture is not only a method to demonstrate chondrogenic potential of stem cells and an effective tool to restore chondrogenic phenotype to expanded cells, but also a method to engineer a spectrum of cartilaginous tissues from a single cell source, thus improving the translational potential of engineered cartilage.

4.6 Alternate Cell Sources. The limited supply of native chondrocytes and their tendency to dedifferentiate could limit their use for tissue engineering, which prompted the investigation of alternate cell sources. Gene expression analysis showed that articular chondrocytes dedifferentiate immediately upon passage. This was evident based on the down-regulation of cartilage-specific genes, such as collagen type II and superficial zone protein, and up-regulation of genes like collagen type I (Fig. 11) [89]. The rapid loss of phenotype in articular chondrocytes suggested that significant problems exist at the front end of tissue engineering efforts. To address this problem, various alternate cell sources, including human embryonic stem cells (hESCs), have been investigated for cartilage tissue engineering. Once practically relevant cell sources can be identified and employed in a comprehensive tissue engineering effort, the as-of-yet intractable problem of cartilage afflictions can be addressed in a functionally useful manner.

4.6.1 hESCs can be Used for Cartilage Tissue Engineering. As embryonic stem cells are multipotent and can proliferate indefinitely, they offer a potentially plentiful cell source. However, it is necessary to control their differentiation toward the chondrogenic lineage. A modular approach, employing chondrogenic differentiation followed by tissue engineering using the self-assembling process, was developed for in vitro cartilage engineering with hESCs (Fig. 12).

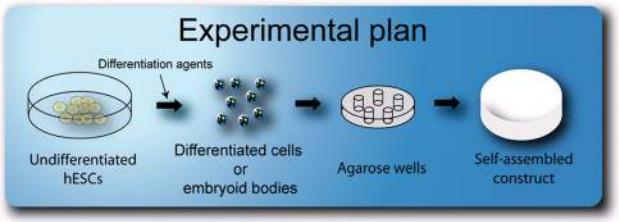

Fig. 12 The modular approach consists of chondrogenic differentiation of hESCs, followed by tissue engineering of the chondrocyte-like cells

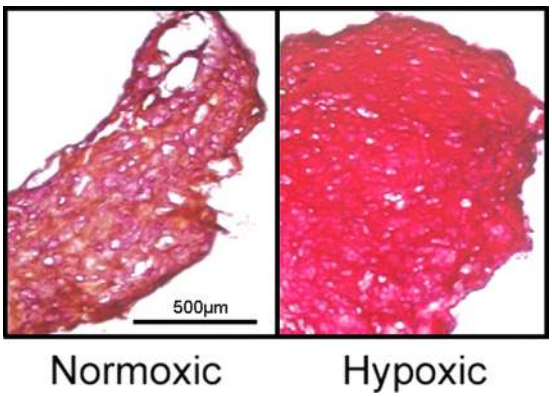

Fig. 13 Hypoxic conditions increased staining for collagen, indicating increased chondrogenesis. (Figure adapted from Koay and Athanasiou [90]).

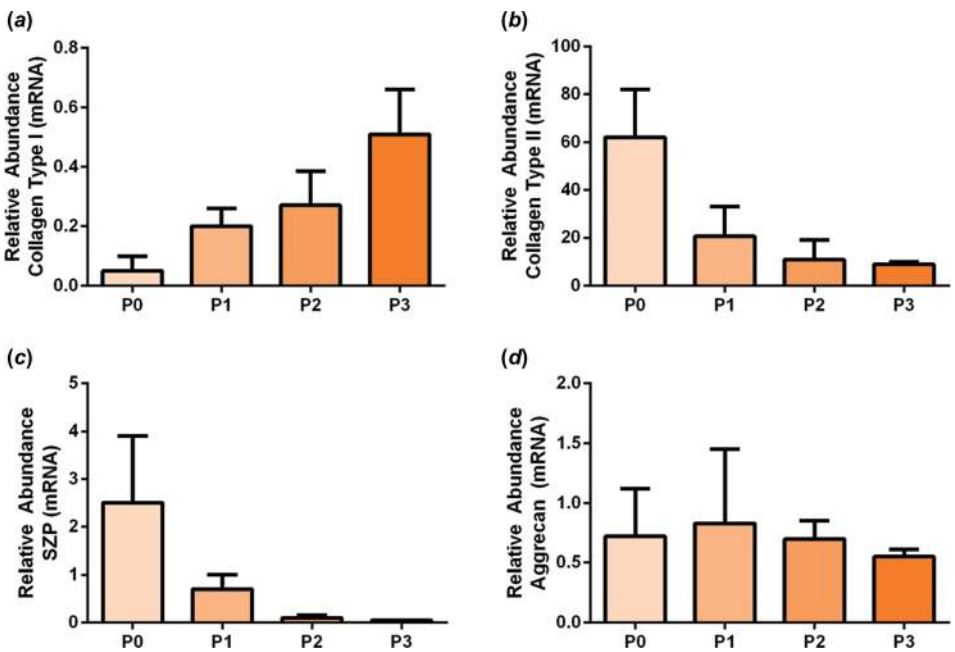

Fig. 11 Collagen type I, which is not normally expressed in cartilage, becomes more common as articular chondrocytes are passaged. In contrast, collagen type II and superficial SZP expression decreases precipitously after 1–2 passages, while aggrecan expression remains relatively constant. (Figure adapted from Darling and Athanasiou [89]).

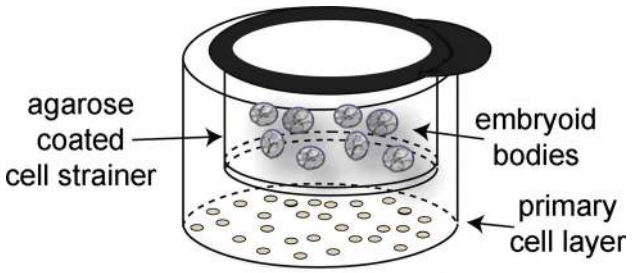

Fig. 14 Using coculture with a fibrochondrocyte feeder layer results in improved chondrogenic differentiation of hESCs. (Figure adapted from Hoben et al. [91]).

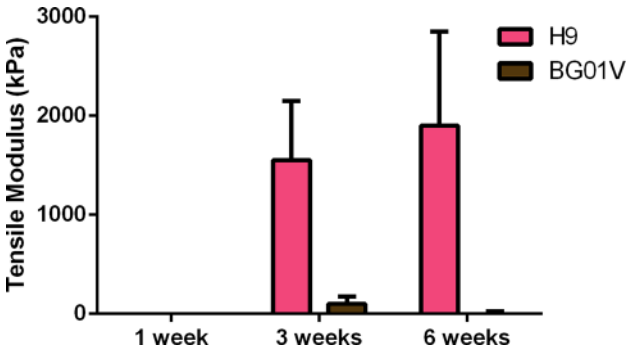

Fig. 15 Cell line dramatically affects neocartilage tensile properties. (Figure adapted from Hoben et al. [94]).

Hypoxic conditions and growth factors have been employed to enhance chondrogenic differentiation. For example, hypoxia (Fig. 13) enhanced the production of collagen type II (3.4 times), collagen type I (2.9 times), and GAGs (1.9 times) [90]. These results showed that oxygen availability had dramatic effects on the differentiation and synthetic potentials of hESCs and could have important implications for the development of strategies to engineer both fibrous and articular cartilages. The combination of TGF-β3 and BMP-4 increased GAG and collagen content as well as increased the abundance of the cell surface marker CD44 [91]. Another study showed that TGF-β application increased GAG deposition and cartilage matrix protein expression [92].

Cocultures have been shown to control hESC differentiation, including the promotion of chondrogenesis. In addition, coculturing hESCs with fibrochondrocytes (Fig. 14) resulted in a 9.8-fold increase in collagen type II production [91]. Similarly, coculturing hESCs with chondrocytes increased GAG deposition as well as expression of collagen type II and Sox 9 [93]. Cocultures provide a powerful method that could supplement differentiation conditions such as growth factor application.

The effects of differentiation time and cell line, H9 versus BG01V, were examined in self-assembled neocartilage. It was determined that a minimum of 3 weeks of chondrogenic differentiation was necessary. Although compressive properties did not vary between cell lines, tensile properties of H9 neocartilage were 1.56–1.94 MPa versus 32–80 kPa in BG01V neocartilage (Fig. 15) [94]. Although these results showed progress in fibrocartilage tissue engineering, the dramatic differences between hESC lines pose difficulties for the eventual translation of hESC-based technologies [95].

5 Future Directions

Although results thus far show great promise for cartilage tissue engineering, additional work is needed prior to clinical application.

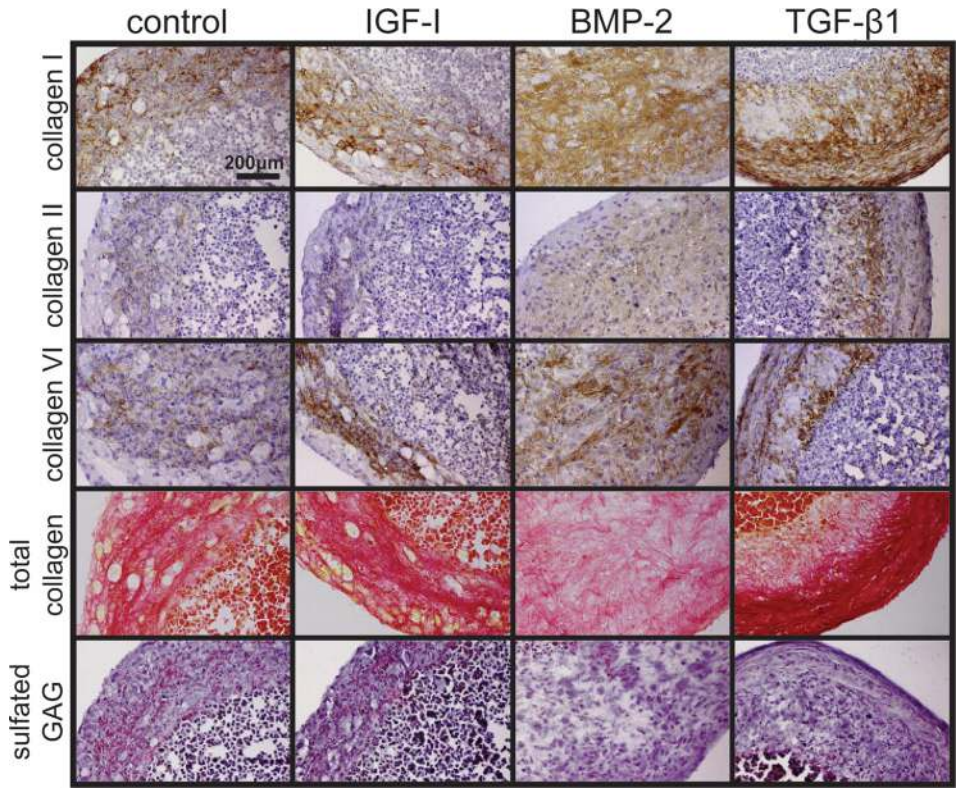

Fig. 16 Histological assessment of DIAS cell neocartilage treated with IGF-I, BMP-2, or TGF-β1, shows staining for collagen type I, II, and VI, indicated chondrogenic ECM content. (Figure adapted from Sanchez-Adams and Athanasiou [96]).

Current studies focus on refining tissue engineering techniques via the application of novel exogenous agents and mechanical stimulation regimens, developing alternate cell sources, and enhancing the integration of neocartilages within native tissue.

5.1 The Potential of DIAS Cells to Form Cartilage. DIAS cells, a population of stem cells natively residing within adult dermis, are a promising cell source for cartilage tissue engineering. They are isolated in a minimally invasive procedure from highly regenerative skin, thus providing an ethically noncontroversial source for potentially autologous tissue engineering therapies [96].

Initial investigation into the use of dermis-derived cells was conducted with dermal fibroblast cell lines. Dermal fibroblasts were expanded and subsequently induced to a chondrocytic phenotype on aggrecan-coated surfaces. After 24 h of culture on aggrecan, dermal fibroblasts formed dense nodules resembling condensing mesenchymal cells. Culturing on aggrecan also increased collagen type II mRNA expression threefold [97]. In a subsequent study, a protocol was developed to isolate a subpopulation that displayed greater chondroinduction potential, termed DIAS cell, from whole dermis by rapid adherence. It was demonstrated that DIAS cells can be used to form cartilage neocartilage, suggesting that skin-derived stem cells can be differentiated to chondrocyte-like cells [98].

Further studies aimed to refine the chondrogenic differentiation and self-assembling conditions of DIAS cells. It was shown via up-regulation of collagen type II and downregulation of collagen type I that DIAS cells selected by rapid adherence were chondrogenically differentiated by passaging in chondrogenic media on uncoated surfaces. DIAS cell neocartilage treated with BMP-2 or TGF-β1 showed enhanced GAG content and compressive properties and similarity to meniscus tissue (Fig. 16). This study also demonstrated the multilineage differentiation potential of DIAS cells by staining cultures for lipids, calcium deposits, and sulfated GAG and collagen type II after culture in adipogenic, osteogenic, and chondrogenic media, respectively [96]. In a separate study, chondrogenic differentiation of DIAS cells under hypoxia resulted in a 2.3-fold increase in collagen type II production per cell and a 1.2-fold increase in GAG content relative to normoxic culture [99].

Encouraged by preliminary success with animal sources of DIAS cells, we now look to adapting chondrogenic differentiation protocols and self-assembling conditions to human DIAS cells. The use of human DIAS cells increases the translatability of tissue engineering work, the exciting potential of which will continue to be explored.

5.2 LOX–Mediated Enhancement of Cartilage Integration. Motivated by the LOX-induced production of collagen crosslinks and increased tensile properties in engineered cartilage, LOX was investigated as an agent to enhance cartilage-to-cartilage integration. To examine this, engineered cartilage and native cartilage explants were press-fitted into defects in native cartilage explants. Increased neocartilage-to-native cartilage interfacial properties, such as tensile stiffness and strength, were

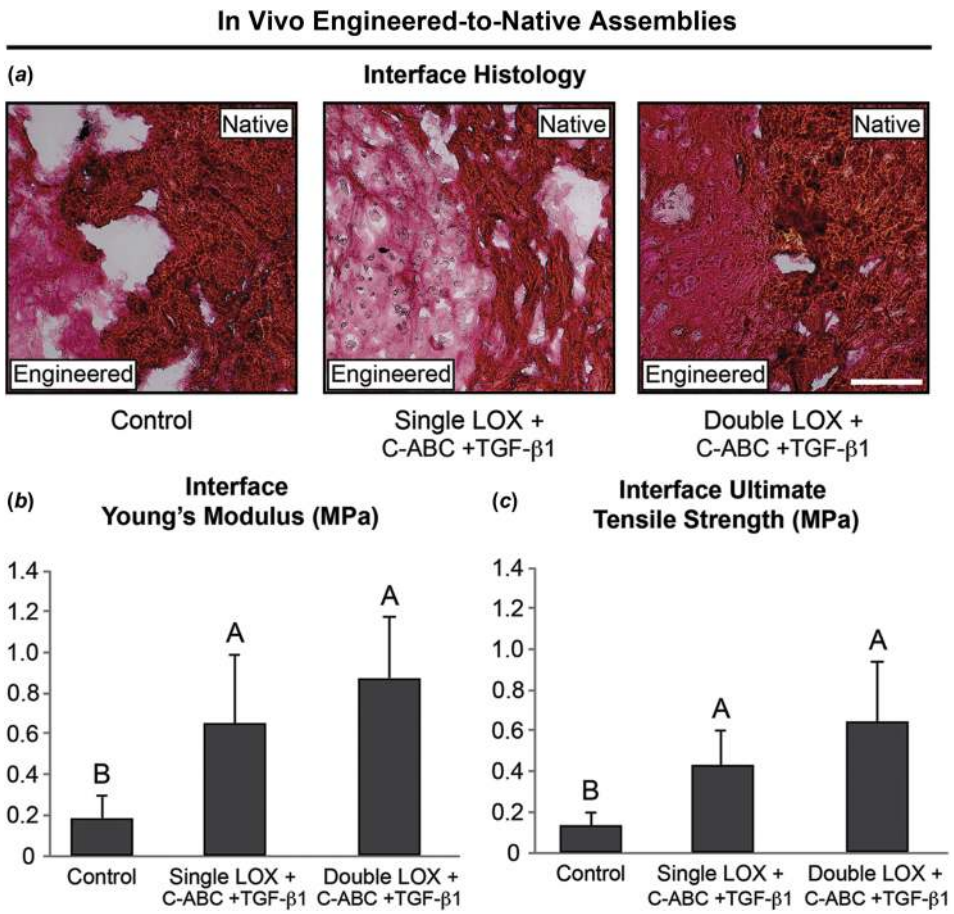

Fig. 17 In Vivo integration interface histological and mechanical assessment shows (*a*) increased tissue integration and collagen staining, (*b*) increased tensile (Young's) modulus, and (*c*) increased interfacial tensile strength in LOX, C-ABC, and TGF-β1 treated groups, specifically the group that received a double LOX treatment. (Figure adapted from Makris et al. [82]).

observed after LOX treatment. Longer durations of treatment resulted in statistically higher interfacial tensile properties, 2.2 times that of the untreated control [100].

A separate study showed that neofibrocartilage treated with LOX, C-ABC, and TGF-β1 is primed for enhanced integration following implantation. The integration interface stiffness of neofibrocartilage and native tissue was significantly increased by 114% and 148%, respectively, in assemblies treated with LOX alone or LOX, C-ABC, and TGF-β1. Additionally, the subcutaneous implantation of native-to-engineered cartilage assemblies pretreated with a two LOX applications and one C-ABC and TGF-β1 treatment increased interfacial Tensile modulus and ultimate tensile strength by 4.3-fold and 4.7-fold, respectively (Fig. 17) [82]. This study showed that LOX treatment primes the integration of highly cellularized and metabolically active neofibrocartilage, a promising approach to the significant obstacle of cartilage integration.

5.3 Osteochondral Tissue Engineering. Cartilage integration with underlying bone may also be approached from the standpoint of engineering the entire osteochondral interface. Unlike cartilage, bone heals and integrates with biomaterials, for example, calcium phosphate ceramics, successfully [101]. Therefore, engineering a replacement tissue, comprising an interdigitated osseous phase and chondral phase, may provide solutions to address graft tissue integration. Specifically, the graft would integrate into native tissue using the osseous phase as an anchorage into subchondral bone. Toward engineering an osteochondral implant, several considerations are to be made: (1) selecting an osseous phase that resorbs at a rate similar to native bone ingrowth, thereby allowing the integration of the implant via natural remodeling processes, (2) the interdigitation of the engineered cartilage phase into the osseous phase of the implant, and (3) the minimization of stress concentrations across the engineered osteochondral interface to allow for the transmission of loads, akin to the function of the native osteochondral interface. Approaches previously shown to improve cartilage mechanical properties, such as application of exogenous stimuli to modulate tissue properties and crosslinking, as well as mechanical stimulation to promote ECM organization, may address these objectives.

6 Conclusions

A biomechanics-driven approach continues to motivate functional cartilage tissue engineering. Cartilage is ultimately a biomechanical tissue, the function of which is to transmit loads. Biochemical properties are concomitant with the mechanical properties and functionality of the tissue. Therefore, biomechanics, and related biochemical properties, will continue to provide the principal success criteria for cartilage tissue engineering efforts.

By leveraging biomechanical characterizations, biomaterials knowledge, and the tools of modern molecular biology and biochemistry, new tissue engineering technologies have continued to emerge. Structure-function characterizations of native tissues serve as design standards for these technologies that, in conjunction with suitable stimuli, are poised to improve tissue regeneration. Future research will continue to examine cartilage biomechanics and regeneration comprehensively, encompassing subcellular to tissue-level studies. This work at the frontiers of cartilage research will help tackle some of the most pressing problems of musculoskeletal medicine. Past successes from our research group and, primarily, from the biomedical engineering field as a whole, guide the path toward addressing the many issues of musculoskeletal tissue engineering including the heretofore intractable problem of cartilage healing.

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