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A Review of In-Vitro Fibrocartilage Tissue Engineered Therapies with a focus on the Temporomandibular Joint

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Abstract

The inability of fibrocartilage, specifically the temporomandibular joint (TMJ) disc, to regenerate and remodel following injury presents a unique problem for clinicians. Tissue engineering then offers a potential regenerative therapy. In vitro testing provides a valuable screening tool for potential tissue engineered solutions. The conclusions drawn for TMJ in vitro research were compared against state of the art fibrocartilage studies in the knee meniscus, and annulus fibrosus of the intervertebral disc (IVD). For TMJ disc regeneration, in vitro tissue engineered approaches, focused on cellular therapies with fibrochondrocytes, have displayed an inability to produce enough collagen, as well as an inability to recapitulate native mechanical properties. Biomaterial approaches have recapitulated the native properties of the TMJ disc, but their in vivo efficacy has yet to be determined. By comparison, the knee meniscus field is the most progressive in the use of stem cells as a cell source. The knee meniscus field has moved away from measuring mechanical properties, and are instead more focused on biochemistry and gene expression. IVD studies mainly use electrospun scaffolds, and have produced the best success in mechanical properties. The TMJ field, in comparison to knee meniscus and IVD, needs to employ stem cell therapies, new biomaterials and manufacturing techniques, and cutting edge molecular assays, in future in vitro approaches to screen for viable technologies to move to in vivo studies.

Keywords

Tissue Engineering; Temporomandibular Joint; Fibrocartilage; In Vitro

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Introduction

Fibrocartilage is the type of cartilage found in temporomandibular joint (TMJ) discs, the annulus fibrosus of intervertebral discs (IVD), and the meniscus of the knee. The clinical necessity of investigating fibrocartilage is highlighted by the large numbers of individuals affected by degeneration of these joints. It is estimated that 10 million Americans are affected by temporomandibular joint disorders, as many as 5 million people are affected by lower back pain attributed to IVD degeneration (Sherman et al., 2010), and 600,000 knee surgeries are performed per year in the United States (Sweigart & Athanasiou, 2001).

Fibrocartilage differs from hyaline and articular cartilage in the ratio of type I collagen to type II collagen (Figure 1). While articular cartilage is predominately collagen type II, fibrocartilage tissues as a group have higher collagen type I content, although the exact ratio can vary by tissue. The TMJ disc, for example, is almost 100% collagen type I (Anderson & Athanasiou, 2009), with trace amounts of collagen type II located in the intermediate zone. The knee meniscus has a heterogeneous distribution of collagen type I and type II, with the lateral head containing almost 100% collagen type I, while the medial head has a collagen I/II ratio of 0.6 (Cheung, 1987). The IVD also has heterogeneous distribution of collagen, with the inner and outer annulus fibrosus having collagen I/II ratios of 0.68 and 0.84, respectively (Eyre & Muir, 1976). These phenotypic differences between the TMJ and both the knee menisci and IVD can necessitate separate treatment modalities for the different tissues.

While current treatments exist for these distinct fibrocartilages, they generally rely on surgical methods that do not restore the original tissue. This is because the avascular nature of these fibrocartilage tissues does not promote healing on its own. As a result, researchers are turning to tissue engineered, cell based therapies as potential grafts for healing and remodeling.

The purpose of this review is to elucidate the progress of TMJ in vitro tissue engineering initiatives and compare to more advanced fibrocartilage fields, such as the IVD and knee meniscus. Recent studies will be reviewed here that focused on biochemical or biomechanical parameters. However, the fields are moving to novel biochemical and molecular biological techniques to analyze smaller quantities of the regenerated tissues. The first part of this review will focus on tissue engineered strategies for TMJ disc remodeling (Table 1). The second part will compare the TMJ disc to advances in knee meniscus (Table 2) and the annulus fibrosus of the IVD tissue engineering (Table 3). It is important to note that selection of the most recent studies precludes the reader from seminal works and the progression of the fields. This relevant data and knowledge can be found elsewhere (Aryaei, Vapniarsky, Hu, & Athanasiou, 2016; Athanasiou, Responde, Brown, & Hu, 2015; Chen, Duan, Zhu, Xiong, & Wang, 2014; Goldberg, Mitchell, Soans, Kim, & Zaidi, 2017; Gugjoo, Amarpal, Sharma, Aithal, & Kinjavdekar, 2016; Kazemnejad, Khanmohammadi, Baheiraei, & Arasteh, 2017; Scotti, Hirschmann, Antinolfi, Martin, & Peretti, 2013; Yu, Adesida, & Jomha, 2015). However, none have directly compared in vitro regenerative medicine approaches for the TMJ disc to other fibrocartilages.

The three major biomechanical components analyzed in this review are collagen, GAGs, and DNA. Collagen is the primary biochemical constituent that imparts tensile strength to the tissue in vivo. Therefore, the recapitulation of the native composition and alignment of collagen can be necessary for a regenerative therapy to reproduce similar tensile properties to native tissues. GAGs are highly negatively charged branched molecules attached to proteoglycans. The negative charge attracts water, which allows GAGs to resist fluid flow and increases the compressive integrity of a tissue. DNA is reported where applicable to demonstrate the general cellularity of proposed therapies, since fibrocartilage is generally considered an acellular tissue.

Temporomandibular Joint Disc

The TMJ disc is a fibrocartilage disc (Figure 2) that is positioned between the mandibular condyle and glenoid fossa of the temporal bone. During normal physiological function, the mandibular condyle slides anteriorly along the inferior surface of the TMJ disc, with the disc shielding the articular eminence from bone on bone contact. The native TMJ disc is composed of 24%/WW (wet weight) collagen and 0.6%/WW glycosaminoglycan (GAG) content (Kalpakci, Willard, Wong, & Athanasiou, 2011). The collagen is aligned in a ring around the periphery of the disc, and is aligned anteriorly-posteriorly throughout the intermediate zone of the disc. The TMJ disc produces tensile forces that are an order of magnitude higher (MPa) than compressive forces (kPa). It is important for any tissue engineered material to exhibit compressive and tensile strengths similar to those observed in the in vivo environment. Otherwise, the regenerative materials may rupture once implanted and require further intervention. We have chosen to review 12 recent publications on TMJ tissue engineering studies performed in vitro in the last 5 years (Table 1). The studies are presented chronologically, and the values reported are compared against native properties of the human TMJ disc (Kalpakci, Willard, et al., 2011).

Anderson and Athanasiou in 2008 analyzed the effect of costal chondrocyte passage number on fibrocartilage tissue engineering (Anderson & Athanasiou, 2008). In this study, 2 million goat TMJ disc or costal cartilage cells were allowed to self-assemble in a scaffoldless approach. TMJ disc cell constructs produced 5 times less collagen (5%/WW) than native, which was more than costal chondrocytes at all passages. Also, TMJ disc cell constructs produced 10 times lower compressive (190 kPa compressive modulus) than native, 11 times lower ultimate tensile strength (UTS) (0.5 MPa) than native, and 16 times lower tensile modulus (2.28 MPa) than native (Table 1). All of these values were significantly higher than the other groups. However, these constructs were prohibitively smaller in size than costal chondrocyte constructs. This study is important because it attempted to establish a standard passage number for in vitro experiments, to ensure continuity among experiments and determine effects. This study suggested the efficacy of the costal chondrocytes, while demonstrating that TMJ disc cells would not be an appropriate cell source, even though these constructs produced greater biochemical components and withstood more mechanical forces.

In an effort to determine how growth factors could impact tissue engineered constructs, Johns and Athanasiou in 2008 investigated the effects of growth factors on cells for

fibrocartilage tissue engineering (Johns & Athanasiou, 2008). In this study, 2 million goat costal cartilage cells were allowed to self-assemble in a scaffoldless approach. These constructs were cultured in DMEM with 1% ITS. The effect of transforming growth factor (TGF), insulin growth factor (IGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) were investigated individually on the cultured cells, along with a non-growth factor control. After 6 weeks of culture, biochemically, the study found IGF produced the best results, with GAG production being 50 times that of native (81%/WW) and collagen production 2 times less than native (14%/WW). The best mechanical properties are achieved by the non-growth factor control, with tensile strength 20 times less than native (0.22 MPa tensile UTS) and modulus 60 times less than native (0.54 MPa tensile modulus) (Table 1). The paper supports that IGF has the best results based on total amount of biochemical constituents. However, if the percentage of GAGs and collagen per wet weight is used, TGF performs the best of the growth factors. Also, the study uses growth factors at varying concentrations (10 mM to 100 mM) depending on the growth factor, making it difficult to compare the groups.

Wang et al in 2009 also investigated cell type for TMJ tissue engineering applications (Wang, Lazebnik, & Detamore, 2009). In this study, porcine hyaline cartilage and mandibular condylar cartilage cells were seeded at 50 million cells/ml scaffold onto non-woven PGA mesh. The scaffolds were cultured in DMEM with 10% FBS. Each cell type was also exposed to D-glucosamine 6-sulfate and IGF, both individually and in combination. After 6 weeks of culture, the hyaline cartilage cells outperformed the mandibular condylar cartilage cells in total GAG and collagen content. The IGF groups had the highest content of GAG (160 µg) and hydroxyproline (2.6 µg). The authors of this paper did not provide the biochemical values as percentages of the wet weight though, so a comparison to native properties or other studies is not possible.

Anderson and Athanasiou in 2009 examined the difference between using primary or passaged chondrocytes when compared to costal chondrocytes as a cell source for fibrocartilage tissue engineering (Anderson & Athanasiou, 2009). In this study, 2 million goat primary (P0) and passaged (P3) articular or costal chondrocytes were allowed to self-assemble in agarose wells. Cells were cultured in DMEM with 1% ITS. After culturing for 4 weeks, it was determined that passaged costal chondrocytes performed the best when compared to all the groups in biochemical assessments, producing 40 times the GAG concentration of native (5%/WW) but 35 times less collagen than native (0.7%/WW). Like costal cells, chondrocytes that were passaged also performed better than primary cells in mechanical properties. These constructs, however, had 5 times less ultimate tensile stress (0.5 MPa) and 25 times less tensile modulus (0.9 MPa) than native (Table 1). This is one of the first studies to show that costal cells can produce fibrocartilage constructs more effectively than hyaline articular cartilage cells.

Kalpakci et al in 2011 studied the effect of TGF β 1 and IGF-1 on fibrocartilage tissue engineering constructs (Kalpakci, Kim, & Athanasiou, 2011). In this study, they used scaffoldless co-cultures of bovine femoral chondrocytes and menisci fibrochondrocytes at different ratios (100:0, 75:25, and 50:50 fibrochondrocytes to femoral chondrocytes). To create the constructs, 5.5 million cells were placed into agarose wells. In DMEM, the effect

of varying concentrations of TGF- β 1 and IGF-1, both individually and in combination, was tested. This study also compared media types, with one set of conditions receiving 10% FBS, and one set receiving no serum treatment. After 4 weeks of culture, the authors performed compressive, tensile, and biochemical assessments. It was determined that TGF- β 1 alone without serum had the largest effect on biochemical production and mechanical properties. These constructs had 20 times the GAG content as the native disc (11%/DW), and two times less collagen content (11%/DW). Also, they were similar to native discs in compressive modulus (0.8 MPa), and were an order of magnitude lower in tensile modulus (1.9 MPa) (Table 1). At the time, this combination produced the highest compressive modulus from in vitro experimentation, as well as the second most collagen from all the studies.

The most recent study to investigate a homogenous cell source in scaffoldless constructs was Hagandora et al in 2012, who examined the effect of magnesium ions on potential tissue engineered fibrocartilage constructs (Hagandora, Tudares, & Almarza, 2012). In this study, 2 million goat costal chondrocytes were allowed to self-assemble in agarose wells. Cells were cultured in DMEM/high glucose with 10% FBS. To the media, varying concentrations of $MgCl_2$ and $MgSO_4$ were added and tested. After culturing for 4 weeks, it was determined that low concentrations of magnesium were not different than control in biochemical or biomechanical assays, and higher concentrations of magnesium were significantly lower. The constructs also had a compressive modulus 4 times less than that of native (450 kPa) (Table 1). All groups produced ten times less collagen than native (7%/DW) and 5 times more GAG content than native (16%/DW). A major limitation to this study is the inclusion of media with serum. Since magnesium is a part of signaling pathways, its contributing effect could be masked by the serum. It would be interesting to see what effect magnesium has on cells without serum to determine if magnesium has any effect.

MacBarb et al in 2013 attempted to engineer scaffoldless fibrocartilage constructs resembling the TMJ disc using shape specific negative molds (MacBarb, Chen, Hu, & Athanasiou, 2013). Specifically, they created 50:50 co-cultures of bovine meniscus and hyaline cartilage cells. 12 million cells were placed into agarose wells with a mold that imparted a biconcave shape and were cultured for 5 weeks. During that time, groups were exposed to either biomechanical (passive axial compression) and/or biochemical (Chondroitinase ABC + TGF- β 1) stimulation. After culturing, it was found that a combination of biochemical and biomechanical stimulation had the greatest effect on mechanical and biochemical properties. The study was also able to produce construct anisotropy, as there were significant differences between the middle zone and periphery of the construct in mechanical testing. The tensile ultimate stress was 5 times less than native (2.3 MPa), while the tensile modulus was an order of magnitude lower than native (3.4 MPa) and the compressive modulus was half of native (900 kPa). In terms of biochemistry, the constructs contained 12 times less collagen content than native (2%/WW) and 12 times more GAG content than native (7%/WW). The production of a shape-specific construct featuring anisotropy in this study is a significant step forward in in vitro research, since it more closely models what is found in vivo.

Following up on the previous study, MacBarb et al in 2013 studied the effect of Chondroitinase ABC and TGF- β on fibrocartilage tissue engineering constructs (MacBarb, Makris, Hu, & Athanasiou, 2013). Specifically, they used co-cultures of bovine articular chondrocytes and meniscus cells at ratios of 50:50 and 75:25 cultured in a scaffoldless technique. In this study 12 million cells were placed into an agarose well and were allowed to self-assemble into a scaffoldless construct. In DMEM with no serum, different combinations of Chondroitinase ABC and TGF- β were tested. After 5 weeks of culture it was found that the combination of Chondroitinase ABC and TGF- β yielded constructs that were 5 times less than native in UTS (2.5 MPa) (Table 1). In terms of biochemical content, the constructs had five times the GAG content as the native disc (7%/WW), but had 6 times less the collagen content (4%/WW).

Chahine et al. in 2014 studied the effect of single-wall carbon nanotubes (SWNTs) for tissue engineering of articular cartilage (Chahine, Collette, Thomas, Genetos, & Loots, 2014). Specifically, they seeded bovine knee articular chondrocytes onto a hydrogel of SWNTs and 2% agarose at a concentration of 10,000,000 cells/mL. After 5 weeks of culture in chondrogenic media, it was determined that SWNTs functionalized with terminal carboxylic acid (COOH) produced a compressive modulus that was 50 times lower than native (45 kPa). In terms of biochemical content, it was determined that SWNTs functionalized with covalent polyethylene glycol (PEG) produced GAG content that was similar to native (0.5%/WW). This paper was unique in its use of carbon SWNTs, and provides a creative alternate material to the other studies presented here. The tensile properties would be important to determine, however, as well as the SWNT's ability to propagate collagen content.

Murphy et al. in 2015 created an ex-vivo defect model that was filled with in vitro neocartilage and determined the effects of biochemical stimulation and enzymatic treatment on neocartilage-TMJ fibrocartilage integration (Murphy, Arzi, Prouty, Hu, & Athanasiou, 2015). Specifically, they cultured porcine costal cartilage at 750,000 cells/mL in a 48 well plate. After 4 weeks of culture, they implanted the resulting neocartilage into a 5 mm defect in a native TMJ disc, and cultured again for 8 weeks. During this time, the constructs were given either no treatment, biochemical treatment (C-ABC and TGF- β 1), or biochemical and enzymatic treatment (media supplemented with lysyl oxidase homologue 2, copper sulfate, and hydroxylysine). After culturing, the authors performed biochemical, compressive, and tensile assessments. It was found that a combination of biochemical stimulation and enzymatic treatment had the greatest effect on mechanical and biochemical properties. The tensile stress (2.3 MPa) was 5 times less than native, and the tensile modulus (6.2 MPa) was 6 times less than native. The compressive modulus (1243 kPa) was half of the native value. For biochemistry, the GAG content (7.5%/WW) was 12 times higher than native, and the collagen content (4%/WW) was 6 times less than native.

Juran et al. in 2015 investigated the use of laser micropatterning on decellularized temporomandibular joint discs as a potential fibrocartilage regenerative therapy (Juran, Dolwick, & McFetridge, 2015). Specifically, the authors excised porcine native TMJ discs and decellularized them with a detergent. After micropatterning the intermediate zone of the decellularized discs, the scaffolds were seeded with Wharton's Jelly-derived MSCs at 900 cells/mm². After 3 weeks of culture, micropatterned scaffolds produced a compressive

modulus that was 1.5 times higher than native (3 MPa). The micropatterned scaffolds also displayed the ability to support cell adhesion, with a cell count of 400,000 cells/scaffold. The innovative use of micropatterning on decellularized fibrocartilage allows for the ECM components that are present in the native TMJ can be retained, removing the need for implanted scaffolds to produce ECM constituents.

Tarafder, et al in 2016 studied the use of micro-precise 3D printed spatiotemporal delivery systems embedded with PLGA microspheres and growth factors (Tarafder et al., 2016). Specifically, MSCs from either human bone marrow (100,000 cells/mL) or rabbit TMJ synovium (200,000 cells/mL) were seeded onto a 3D printed scaffold with a similar anatomical shape and contour to a native TMJ disc. These scaffolds were also embedded with or without connective tissue growth factor (CTGF) and TGF- β 3. After 6 weeks of culture, the scaffolds embedded with growth factors produced 20 times less collagen (1.4% collagen/WW), and 4 times more GAG (2.3% GAG/WW). While the biochemical content of the scaffolds did not recapitulate native properties, the 3D printed scaffolds were able to produce densely aligned fibrous matrix in the anterior and posterior band of the scaffold. The success with collagen alignment is a promising step forward for in vitro fibrocartilage regeneration.

Based on the in vitro studies that have been performed, the highest amounts of ECM produced, GAGs and collagen, were obtained when TGF- β 1 was used in the culture media. The literature suggests that scaffoldless constructs outperformed seeded-scaffold constructs. However, there has not been a recent direct comparison between the scaffoldless approach and cutting-edge polymer scaffolds. While progress has been shown, current in vitro methods all produce biomechanical and biochemical properties lower than the native TMJ disc. The largest difference for biomechanical properties comes in the tensile modulus, which is often an order of magnitude lower than native. For biochemical production, studies using fibrochondrocytes often do not produce enough collagen and produce too much GAGs content when compared to native TMJ tissues. in vitro Therefore, moving forward it may be appropriate to investigate the efficacy of stem cells as a cell source for TMJ fibrocartilage tissue engineering. In the other fibrocartilage tissue engineering fields, stem cells have already become the gold standard for achieving closer tissue properties to native.

Other Fibrocartilage: Knee Meniscus and Annulus Fibrosus

Knee Meniscus

The knee meniscus is formed by the medial and lateral fibrocartilage heads in the knee joint (Figure 3). The knee meniscus is composed of two fibrocartilage structures located laterally and medially between the tibial plateau and the femoral condyles. Physiologically, the meniscus withstands compressive and tensile loads, with the medial meniscus bearing 50% of the load, while the lateral meniscus bears up to 70% of the load (Walker PS, 1975). The native knee meniscus is composed of 75%/DW (wet weight) collagen and 2%/DW GAG content. Due to the advanced nature of knee research in comparison to the TMJ field, we decided to highlight stem cell in vitro research, since the field has generally moved beyond native tissue cell sources for tissue engineering purposes. To provide a brief overview of recent advances in knee meniscus fibrocartilage research, the search for papers in the knee

meniscus area were focused on the 5 most recent in vitro papers that contained biomechanical/biochemical results for comparison (Table 2). These papers will be presented chronologically, and compared against the human native properties of the knee meniscus presented by Sweigart and Athanasiou (Sweigart & Athanasiou, 2001). For a more in depth, detailed review of knee meniscus tissue engineering literature, other reviews are more appropriate (Goldberg et al., 2017; Scotti et al., 2013).

Matthies et al in 2013 examined the effect of co-culturing stem cells with native cells on matrix formation for fibrocartilage tissue engineering (Matthies, Mulet-Sierra, Jomha, & Adesida, 2013). In this study, 250,000 human bone marrow stem cells (BMSCs) and human meniscus cells were allowed to self-assemble in co-cultures of various ratios. Cells were cultured in DMEM with 1% ITS and TGF- β 3. The scaffolds were also conditioned with either normal or lowered oxygen tension. After 3 weeks of culture, it was determined that co-cultures of BMSCs and meniscus cells at a ratio of 75:25 performed the best for matrix formation at lowered (3%) oxygen (20 μ g GAG/ μ g DNA) when compared with other co-cultures and pure BMSC and meniscus cultures, but the units prevent comparison to native. While the use of co-cultures proposed in this paper appears promising, the use of meniscus cells indicate a future requirement of two surgeries to prepare the meniscus, instead of only one.

Chowdhury et al in 2013 investigated the efficacy of chemical treatments on stem cells and meniscus cells (Chowdhury, Bezuidenhout, Mulet-Sierra, Jomha, & Adesida, 2013). In this study, 250,000 human meniscus cells and MSCs were allowed to self-assemble, both individually and in co-culture. Cells were cultured in DMEM with 10 ng/mL TGF- β 1. The authors also chemically treated some scaffolds from each group with 500 pg/mL interleukin-1 β . After 17 days of culture, interleukin-1 β had no effect on overall synthesis of GAGs, with no significant differences being seen when comparing similar pellets with or without chemical treatment. Co-culturing BMSCs and meniscus cells produced similar results with BMSCs alone, producing 6.3 μ g GAG/ μ g DNA. Both groups produced GAG significantly more than meniscus cells individually. This paper further advanced the theory that co-cultures of stem cells with meniscus cells provides a potential solution for fibrocartilage tissue engineering.

Croutze et al. in 2013 investigated the effect of oxygen concentration on the biochemistry of inner and outer human meniscus cells on a 3D collagen scaffold (Croutze, Jomha, Uludag, & Adesida, 2013). In this study, the authors seeded human meniscus cells at 1 million cells/scaffold at passage 3 onto collagen scaffolds. The scaffolds were allowed to culture for 3 weeks in 3% or 21% oxygen. After 3 weeks, it was determined that the 21% oxygen group showed the highest GAG content (23 μ g/ μ g DNA). Also, the authors showed that scaffolds seeded with meniscus cells from the outer meniscus produced higher GAG content (23 μ g/ μ g DNA) than inner meniscus cells (18 μ g/ μ g DNA). The GAG content puts this study on the low end of the studies reviewed here, but also highlights the importance of culture conditions and tissue harvest location on tissue engineering efficacy.

Fisher et al in 2015 investigated the functionality of single and multi-layered MSC-seeded nanofibrous scaffolds (Fisher et al., 2015). Specifically, they engineered PCL nanoscaffolds

via electrospinning, and organized the fibers in various angled orientations. Fibers were either arranged in parallel with the direction of the meniscus (0°), perpendicular to the meniscus (90°) or circumferentially. For multilayered scaffolds, combinations of these directions were combined. The scaffolds were then seeded with bovine MSCs at a concentration of 500,000 cells per side of a scaffold and allowed to culture for 6 weeks of culture. In terms of tensile modulus, single layer scaffolds in the 0° direction achieved the highest value (22 MPa), which was roughly the same as native. For collagen content, the multi-layered scaffolds with three sheets in the 0° direction provided the highest collagen content (60 ug). Since the authors only provide total content and no scaffold weights, this cannot be compared to native. This experiment provided an interesting tunable scaffold for tissue engineering, since you can use the directionality and number of nanofibrous layers to achieve higher or lower mechanical and biochemical properties.

Visser et al in 2015, attempted to develop crosslinkable hydrogels for cartilage tissue engineering from cartilage, knee meniscus, and tendon tissue (Visser et al., 2015). Specifically, they harvested femoral condylar cartilage, knee meniscus, and the patellar tendon from horses, and each tissue was decellularized and mixed with gelatin methacryloyl (GelMA) and crosslinked to create hydrogels. It was determined that the cartilage hydrogel achieved the highest compressive modulus (66.2 kPa), which is 10 times less than native. While this scaffold initially displays lower compressive modulus, it would be expected that when seeded with cells, the compressive properties would increase as infiltrating cells began depositing matrix. The authors seeded equine MSCs and chondrocytes on the scaffolds as well, but only reported normalized biochemical results, making these results incomparable to the other studies presented here.

From the papers reviewed, the lack of mechanical testing performed on the proposed tissue engineered scaffolds makes it difficult to compare to different fields. A similar problem of comparison is observed in the lack of collagen content reported. From the provided GAG data alone, however, it appears that the tissue engineered therapy most suited to producing GAG is the SIS-ECM device seeded with meniscus cells and synovium-derived stem cells, studied by Tan et al. In a broader view of the field, it appears that the trend is moving towards more chemical stimulation of tissue engineered scaffolds in lieu of mechanical stimulation.

In comparison with the TMJ field, the compressive properties achieved by the knee meniscus field are an order of magnitude lower than those obtained in the TMJ field. This suggests that the various chondrocytes and fibrochondrocytes used in TMJ tissue engineering are better suited for producing extracellular matrix components with high compressive properties than stem cells. However, in tension, both fields have achieved high properties. For biochemistry, it is harder to compare the fields due to the wide range of units reported, but it appears that in both fields collagen production is substantially lower than native, while GAG production is elevated over native. Source of autologous fibrochondrocytes will be a major hurdle for current TMJ tissue engineering approaches. As such, the field will have to move to stem cell sources or acellular therapies.

Intervertebral Disc

The intervertebral disc consists of two separate sections, the annulus fibrosus and the nucleus pulposus (Figure 4). The annulus fibrosus forms an outer ring that surrounds the gel-like nucleus pulposus. The annulus fibrosus is comprised of several laminae of fibrocartilage, and is the focus for this review. The primary physiological purpose of the IVD fibrocartilage is to resist compressive forces imparted from the spine. The native knee meniscus is composed of 54%/DW (wet weight) collagen and 6%/DW GAG content. The increased GAG content and reduced collagen content highlight how the annulus fibrosus' primary function is its compressive strength. Like the knee meniscus field, the research being performed in the knee meniscus field is much more varied than the TMJ. However, similar to knee research, there were limited studies that reported biomechanical or biochemical results. For this review, we limited the review to the last 5 in vitro papers that contained biomechanical/biochemical results for comparison (Table 3). These papers will be presented chronologically, and compared against native human annulus fibrosus biochemical properties from Best et al, native compressive properties from Freeman et al, and averaged tensile values from Issac et al and Stemper et al (Best et al., 1994; Freeman, Buttermann, Beaubien, & Rochefort, 2013; Isaacs, Vresilovic, Sarkar, & Marcolongo, 2014; Stemper, Baisden, Yoganandan, Shender, & Maiman, 2014).

From the same research group as Turner et al, Iu et al in 2014 investigated the use of inner and outer annulus fibrosus cells on a polycarbonate urethane (POU) scaffold (Iu, Santerre, & Kandel, 2014). Specifically, they seeded bovine inner or outer annulus fibrosus at a concentration of 21,000 cells/mm² onto PU scaffolds and allowed for 2 weeks of culture. It was determined that both groups produced tensile moduli of 25 MPa on par with native, and ultimate tensile stresses of 6.5 MPa, which were 2 times native. While ultimately there proved to be no differences in mechanical properties, this was the first study to investigate the idea of comparing annulus fibrosus cell location.

Wisner et al in 2014 explored the use of various scaffold structures and compositions to determine the best option for annulus fibrosus tissue engineered repair (Wisner et al., 2014). Specifically, they seeded cow annulus fibrosus cells at 79,000 cells/cm² onto PU or polycaprolactone (PCL) scaffolds. These scaffolds were either processed as oriented or non-oriented electrospun scaffolds. After 3 weeks of culture, it was determined that both unoriented PU and PCL scaffolds produced the same DNA content (12.5 µg) and GAG content (62 µg), higher than the other scaffolds. The oriented PCL scaffolds, however, produced the highest collagen content (41 µg/scaffold), although this was not a significant difference. In biomechanics, the oriented PU scaffold produced the tensile stress (7 MPa) that was 2 times native, while the oriented PCL scaffolds produced the tensile modulus (55 MPa) that was 2 times native. The fact that each scaffold was optimal for different properties allows researchers to tune the scaffold of choice to the design criteria they feel is most important. Orientation of the scaffold fibers appears to be most important for biomechanical properties, while a lack of orientation appears to favor biochemical properties.

Guillaume et al in 2014 investigated the effects of chemical stimulation and oxygen concentration on annulus fibrosus-seeded porous alginate scaffolds (Guillaume et al., 2014). Specifically, they seeded 800,000 porcine annulus fibrosus cells/mL onto a cross-linked

porous alginate scaffold and allowed them to culture for 3 weeks. The scaffolds were cultured either with or without TGF- β 3 and in either 5% or 20% oxygen. It was determined that scaffolds that were chemically treated with TGF- β 3 and 20% oxygen produced the highest DNA (40 ng/mg). The TGF- β 3 treated groups in low oxygen conditions produced the highest GAG concentration (60 μ g/ μ g DNA), and collagen content (2 μ g/ μ g DNA). Oxygen had little effect on biomechanical properties, as both low and high oxygen TGF- β 3 groups produced a peak compressive stress of 1 kPa and compressive modulus 7 kPa. These values were compared to native porcine AF tissue at the same testing conditions in the study, and they were 0.7 times native for both values. This varies greatly from the native values listed in Table 3, indicating the viscoelastic nature of this tissue based on the different strain rates used.

Colombini et al in 2015 performed an in vitro characterization of annulus fibrosus cells in collagen-enriched fibrin gels (Colombini et al., 2015). Specifically, they seeded 600,000 human annulus fibrosus cells/scaffold onto either fibrin gels or collagen-enriched fibrin gels. Gels were then cultured for 24 hours and assessed for biochemical content. It was determined that the collagen-enriched fibrin scaffolds produced 3 μ g GAG/mg tissue, which corresponds to 0.03%/DW, a value that is 60 times less than native. While this appears to be too low for tissue engineered scaffolds, it should be noted that the in vitro result was a small part of a larger in vivo study, and these collagen-enriched fibrin scaffolds actually produced more GAG than native after in vivo implantation.

In another study by Guillaume et al, this time in 2015, they designed a scaffold construct used to fix defects in the annulus fibrosus in-vivo, as opposed to replacing the entire tissue (Guillaume, Naqvi, Lennon, & Buckley, 2015). 425,000 porcine annulus fibrosus cells were seeded onto either alginate or alginate-collagen scaffolds. Each scaffold was treated with either TGF- β 3 or phosphate buffered saline (PBS). After 3 weeks of culture, it was determined that TGF- β 3 loaded alginate-collagen scaffolds produced the highest DNA content, at 1900 ng/scaffold. The authors also investigated cell migration in these scaffolds, and were able to demonstrate that the TGF- β 3 loaded alginate-collagen scaffolds promoted migration of annulus fibrosus cells throughout the scaffold, compared to only migration along the periphery for TGF- β 3 loaded alginate scaffolds. Moving forward, it would be interesting to see if cell-seeded scaffolds outperform the noncellular scaffolds in biomechanical testing.

For in vitro research, the trend appears to be working towards more complex systems that engineer scaffolds incorporating a gel like center and fibrocartilage based outer ring, like the system described in See et al's 2011 study (See, Toh, & Goh, 2011). From the studies reviewed here, there is a lack of investigation into the effects of mechanical stimulation. Previous studies however, not reviewed here, have investigated the impact of mechanical stimulation (Li, Jia, Duance, & Blain, 2011; Reza & Nicoll, 2008; See et al., 2011; Sowa et al., 2015; Tsai, Nelson, Anderson, Zdeblick, & Li, 2014).

It is also clear that the field is heavily focused on the use of electrospun scaffolds. This tendency is understandable due to the layered, laminar nature of the annulus fibrosus in vivo. Also, all 5 studies utilized annulus fibrosus cells as the cell source for their regenerative

approaches. It is surprising that there were not more stem cell studies performed, but perhaps that is the future direction of the field. The lack of comparable units for biochemical results also limits the ability to compare between studies.

In comparison with the TMJ field, the compressive properties achieved for tissue engineered annulus fibrosus replacements were the lowest of any fibrocartilage field. A caveat to this is that the compressive strength of the native annulus fibrosus is a full order of magnitude less (230 kPa) than the native TMJ disc (2085 kPa), so the lower compressive properties would be expected. In tension, the annulus fibrosus devices reviewed here far outperformed the TMJ community, with values exceeding native and either exceeding (tensile stress) or matching (tensile modulus) the TMJ field. Their improved performance could be based on the use of highly aligned electrospun scaffolds. It could be of interest to the TMJ community to attempt studies using these types of scaffolds.

Conclusions

The discussed tissue engineered therapies highlight the recent advances in the fibrocartilage regenerative medicine field. For TMJ disc regeneration, in vitro tissue engineered approaches, focused on cellular therapies with fibrochondrocytes, have displayed an inability to produce enough collagen, as well as an inability to recapitulate native mechanical properties. Biomaterial approaches have recapitulated the native properties of the TMJ disc, but their in vivo efficacy has yet to be determined. By comparison, the knee meniscus field is the most progressive in the use of stem cells as a cell source. The knee meniscus field has moved away from measuring mechanical properties, and are instead more focused on biochemistry and gene expression. IVD studies mainly use electrospun scaffolds, and have produced the best success in mechanical properties. The TMJ field, in comparison to knee meniscus and IVD, needs to employ stem cell therapies, new biomaterials and manufacturing techniques, and cutting edge molecular assays, in future in vitro approaches to screen for viable technologies to move to in vivo studies. Since the nature of in vitro studies are to experiment with new technologies and determine their potential efficacy in vivo, it could be assumed that fields of research with more in vivo studies are further along than those with less studies. Both knee meniscus and IVD have much more in vivo research than the TMJ.

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Highlights

- There are a variety of scaffold methods for in vitro fibrocartilage research.
- Temporomandibular joint in vitro scaffolds do not produce sufficient collagen.
- In vitro knee meniscus studies require more focus on mechanical properties.
- Intervertebral disc studies have progressed to complex systems to mimic in vivo.

Figure 1.

Spectrum of cartilage based on collagen I/II ratio. Fibrocartilage skews towards the higher Col I/II ratio, with the TMJ disc having a Col I/II ratio of almost 1.0. The knee meniscus and IVD have a Col I/II ratio of 0.8. Hyaline and articular cartilage have a Col I/II ratio of near 0.0.

Figure 2.
Rendering of the inferior face of the TMJ. Collagen is aligned anterior-posteriorly in the intermediate zone, and radially around the outer ring of the disc.

Figure 3. Rendering of the knee meniscus. The meniscus is composed of the medial and lateral heads. The medial head has a collagen I/II ratio of 0.6, while the lateral head has a collagen I/II ratio of almost 1.0.

Figure 4. Rendering of the annulus fibrosus. It contains several rings of radially aligned collagen, and has a collagen I/II ratio of 0.8. The center area would contain the nucleus pulposus.

TMJ in vitro tissue engineered therapy studies, describing cell source, scaffold type, species, mechanical properties, and biochemical properties. A blank space means no data was available in the study.

Table 1

Author	Year	Cell Type	Scaffold	Species	Compressive Modulus (kPa)	Tensile UTS (MPa)	Tensile Modulus (MPa)	GAG (%/WW)	Collagen (%/WW)	DNA (million cells)
Anderson & Athanasiou	2008	TMJ disc cells and costal chondrocytes	None	Goat	190	1.1	2.3	10	5	
Johns & Athanasiou	2008	Costal chondrocytes	None	Goat		0.22	0.54	81	14	2.2
Wang et al	2009	Mandibular condylar fibrochondrocytes and articular chondrocytes	PGA	Pig				160 ug (total)	2.6 ug (total)	1.75
Anderson & Athanasiou	2009	Articular and costal chondrocytes	None	Goat		0.5	0.9	5	0.7	1.5
Kalpakci et al	2011	Co-cultured articular chondrocytes and meniscus	None	Cow	800		1.9	11	11	
Hagandora et al	2012	Costal chondrocytes	None	Goat	450			16 (%/DW)	7 (%/DW)	1
MacBarb et al	2013	Co-cultured articular chondrocytes and meniscus	None	Cow		2.3	3.4	7	2	
MacBarb et al	2013	Co-cultured articular chondrocytes and meniscus	None	Cow		2.5		7	4	
Chahine et al	2014	Bovine articular chondrocytes	nanotubes	Cow	45			0.5		
Murphy et al	2015	Costal cartilage	None	Pig	1243	2.3	6.2	7.5	4	
Juran et al	2015	TMJ disc cells	Decellularized TMJ disc	Pig	3000					0.4
Tarafder	2016	Bone marrow stem cells	PLGA microspheres	Human				2.3	1.4	
Native Properties (Kalpakci, Willard, et al., 2011)	2011			Human	2085	12.8	37.2	0.6	24	.08%/WW

Knee mensicus in vitro tissue engineered therapy studies, describing cell source, scaffold type, species, mechanical properties, and biochemical properties. A blank space means no data was available in the study.

Table 2

Author	Year	Cell Source	Scaffold	Species	Compressive Modulus (kPa)	Tensile UTS (MPa)	Tensile Modulus (MPa)	GAG (ug/DNA)	Collagen (ug/DNA)
Mattheis et al	2013	Bone marrow stem cells and meniscus cells	None	Human				21	
Chowdhury et al	2013	Bone marrow stem cells and meniscus cells	None	Human				6.3	
Croutze et al	2013	Outer and inner meniscus cells	collagen	Human				23	
Fisher et al	2015	Mesenchymal stem cells	Nanofibrous PCL	Cow			22		60 µg (total)
Visser et al	2015	None	Decellularized tissue/hydrogel	Horse	66.2				
Native Properties (Sweigart & Athanasiou, 2001)	2011			Human	662	20	24	2 (%/DW)	75 (%/DW)

Intervertebral disc in vitro tissue engineered therapy studies, describing cell source, scaffold type, species, mechanical properties, and biochemical properties. A blank space means no data was available in the study.

Table 3

Author	Year	Cell Source	Scaffold	Species	Compressive Modulus (kPa)	Tensile Modulus (MPa)	Ultimate Tensile Stress (MPa)	GAG (%/DW)	Collagen (%/DW)	DNA (ng/scaffold)
Iu et al	2014	Annulus Fibrosus	Polycarbonate urethane	Cow		25	6.5			
	2014	Annulus Fibrosus	Polycaprolactone	Cow		55	7	62 µg	41 (µg/scaffold)	12.5 µg
Guillaume et al	2014	Annulus Fibrosus	Porous alginate	Pig	7			60 (µg/µg DNA)	2 (µg/µg DNA)	40 ng/mg
Colombini et al	2015	Annulus Fibrosus	Fibrin gels	Human				0.03		
Guillaume et al	2015	Annulus Fibrosus	Alginate-collagen	Pig	6					1900 ng/scaffold
				Human	230	35.5	3.2	6	54	
Native Properties (Best et al., 1994; Freeman et al., 2013; Isaacs et al., 2014; Stemper et al., 2014)										