Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Matrix Biol. Author manuscript; available in PMC 2015 June 19.

Published in final edited form as:

Matrix Biol. 2005 February ; 24(1): 45–57. doi:10.1016/j.matbio.2004.11.006.

Quantitative analysis and comparative regional investigation of the extracellular matrix of the porcine temporomandibular joint disc

Michael S. Detamore[a,*], **John G. Orfanos**[b], **Alejandro J. Almarza**[c], **Margaret M. French**[c], **Mark E. Wong**[b], and **Kyriacos A. Athanasiou**[c]

[a]Department of Chemical & Petroleum Engineering, University of Kansas, 1530 W. 15th St., Room 4132, Lawrence KS 66045-7609, United States

[b]Department of Oral and Maxillofacial Surgery, University of Texas Health Science Center at Houston Dental School, Houston TX 77030, United States

[c]Department of Bioengineering, Rice University, Houston TX 77251, United States

Abstract

Characterization of the extracellular matrix of the temporomandibular joint (TMJ) disc is crucial to advancing efforts in tissue engineering the disc. However, the current literature is incomplete and often contradictory in its attempts to describe the nature of the TMJ disc matrix. The aim of this study was to identify the variation of key matrix components along the three axes of the porcine disc using ELISAs to quantify these matrix components, immunohistochemistry to identify their regional distribution, and SEM to examine collagen fiber diameter and orientation. The overall GAG content of the TMJ disc (including the dermatan sulfate proteoglycans) was 5.3±1.2% of the dry weight. Chondroitin sulfate, which comprised 74% of this total GAG content, was 4.4, 8.2, and 164 times more abundant than dermatan sulfate proteoglycan, keratan sulfate, and hyaluronic acid, respectively. In general, these GAGs were most concentrated in the intermediate zone of the TMJ disc, appearing in dense clusters, and least concentrated in the posterior band. Additionally, chondroitin sulfate was more abundant medially than laterally. Collagen II was discovered in trace amounts, with higher relative amounts in the intermediate zone. Collagen fibers were observed to run primarily in a ring-like fashion around the periphery of the disc and anteroposteriorly through the intermediate zone, with a mean fiber diameter of 18±9 μm. Characterization studies of the TMJ disc, including prior biomechanical and cell studies along with the current study of the extracellular matrix, collectively reveal a distinct character of the intermediate zone of the disc compared to its anterior and posterior bands.

Keywords

Temporomandibular joint; Disc; Disk; ELISA; Immunohistochemistry; SEM

© 2004 Elsevier B.V./International Society of Matrix Biology. All rights reserved.

[*]Corresponding author. Tel.: +1 785 864 4943; fax: +1 785 864 4967. detamore@ku.edu (M.S. Detamore). .

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

1. Introduction

Unlike other musculoskeletal soft tissues, the temporomandibular joint (TMJ) disc (Fig. 1) is still shrouded in mystery owing to the scarcity of both descriptive and quantitative studies. At the forefront of investigations are those examining its native extracellular matrix content and organization, which are directly responsible for its observed mechanical behavior. A thorough analysis of available studies on the extracellular matrix of the TMJ disc has revealed areas where important questions still remain (Detamore and Athanasiou, 2003a,c).

For example, the TMJ disc is known to consist primarily of type I collagen, but is type II collagen, the most abundant component of hyaline cartilage, also present? Collagen II has been detected in human (Kondoh et al., 2003), primate (Mills et al., 1994), bovine (Landesberg et al., 1996), and rat (Fujita and Hoshino, 1989) discs. In contrast, a study using primate TMJ discs did not detect collagen II (Milam et al., 1991). Collagen II has been found primarily in the regions surrounding cells (Fujita and Hoshino, 1989; Kondoh et al., 2003; Mills et al., 1994), and also within the interstices between collagen I fibers (Mills et al., 1994). In sagittal sections of human discs, more collagen II was found near the surface compared to the interior (Kondoh et al., 2003). This same distribution was observed with type II procollagen peptide, indicative of collagen II synthesis (Kondoh et al., 2003). This protein was found exclusively in and around chondrocyte-like cells.

Elastin fibers are known to be integrated with collagen fibers of the TMJ disc, which leads to the next question: how is elastin distributed throughout the TMJ disc? While previous studies agree that elastin is heterogeneously distributed in the disc (Christensen, 1975; Gross et al., 1999; O'Dell et al., 1989, 1990), reports disagree as to its actual distribution. A study of human discs found about 70% of elastin fibers in the anterior band, 25% in the posterior band, and 5% in the intermediate zone (Gross et al., 1999). In contrast, elastin in porcine discs was most concentrated in the posterior band and least concentrated in the intermediate zone (Christensen, 1975). However, these reports do appear to agree that less elastin is present in the intermediate zone. Accordingly, another study found that elastin fibers dramatically increased in number from the center to the periphery (O'Dell et al., 1990).

Several questions exist pertaining to the glycosaminoglycans (GAGs) of the TMJ disc, known to have important functional significance. First and foremost, what fraction of the dry weight do GAGs account for in the TMJ disc? Reports of total GAG content have ranged over an order of magnitude from 0.5% to 10% (Almarza et al., accepted for publication; Axelsson et al., 1992; Nakano and Scott, 1989, 1996; Okazaki et al., 1996; Sindelar et al., 2000). In the middle of this range, 5% GAG dry weight content was reported for bovine TMJ discs using ion-exchange chromatography (Nakano and Scott, 1989) and 3.24% glucuronic acid dry weight content was determined in rat TMJ discs using electrophoresis (Okazaki et al., 1996). Much lower GAG content dry weight values have been reported for the human disc using high-performance liquid chromatography: 0.54±0.10% for the anterior band/intermediate zone and 0.62±0.10% for the posterior band (Axelsson et al., 1992). Also in this range, GAGs were found to account for 0.96±0.39% of the dry weight in porcine TMJ discs (Almarza et al., accepted for publication). On the other end of the spectrum, an examination of total GAG content in porcine discs using a colorimeteric assay of histology

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

samples found GAG contents ranging from 7.2% to 14.4% of the dry weight (Sindelar et al., 2000).

In addition to varying reports on total GAG content, GAG distribution within the TMJ disc has also varied between studies. Regional GAG content was measured in porcine discs, comparing the medial and lateral regions of the anterior band, intermediate zone, and posterior band (Sindelar et al., 2000). In this study, the medial region contained 47% and 14% more GAGs than the lateral region in the anterior and posterior bands, respectively. However, the lateral region of the intermediate zone contained 39% more GAGs than the medial region. In another study, the amounts of individual GAGs were compared between the center and periphery of the bovine disc (Nakano and Scott, 1996). The center of the bovine disc was determined to contain higher amounts of chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid than the periphery. In contrast, large chondroitin sulfate proteoglycans in the rat disc were more concentrated in the periphery compared to the center, and dermatan sulfate proteoglycans were more prevalent in the anterior band and posterior band compared to the intermediate zone (Kuwabara et al., 2002; Mizoguchi et al., 1998).

The above reference to individual GAGs leads to more questions that still remain unanswered. The primary glycosaminoglycans (GAGs) of the TMJ disc are suggested to be chondroitin sulfate and dermatan sulfate, but which is more prevalent? Moreover, do hyaluronic acid and keratan sulfate comprise a significant portion of the GAG population? In a comparison of relative amounts of various GAGs in the bovine TMJ disc, chondroitin sulfate was determined to be the most concentrated GAG in the disc and by far the most abundant GAG in the center of the disc (Nakano and Scott, 1996). Dermatan sulfate was the next most abundant overall, followed by keratan sulfate. In addition, dermatan sulfate was found to be the most abundant GAG in the periphery of the bovine disc. An earlier study by the same group found chondroitin sulfate to comprise 79% of the total GAG content, the remainder being dermatan sulfate (14%), hyaluronic acid (5%), and keratan sulfate (2%) (Nakano and Scott, 1989). An investigation of human discs found that chondroitin sulfate and dermatan sulfate collectively accounted for about 85% of the total GAG content of the disc, with hyaluronic acid, heparan sulfate, and keratan sulfate only accounting for about 10%, 5%, and 0.2% of the total, respectively (Axelsson et al., 1992). These studies would seem to suggest that chondroitin sulfate is the predominant GAG in the TMJ disc. However, the GAGs of rat TMJ discs were found to be 60% dermatan sulfate, 21% hyaluronic acid, 16% chondroitin sulfate, and 3% heparan sulfate (Okazaki et al., 1996). With respect to keratan sulfate, concentrations on a dry weight basis have been reported as 0.1% (Nakano and Scott, 1989), 0.95±0.33% in the center and 0.0022±0.0009% in the periphery (Nakano and Scott, 1996), less than 0.001% (Axelsson et al., 1992), and undetected (Okazaki et al., 1996). Reports of hyaluronic acid concentrations have ranged from around 0.06% (Axelsson et al., 1992; Okazaki et al., 1996) up to about 0.25% (Nakano and Scott, 1989, 1996) of the dry weight.

Assuming that answers were provided to the aforementioned questions, important questions would still remain with regard to how extracellular matrix content and organization is manifested in observed mechanical properties. Specifically, how does collagen fiber

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

orientation affect tensile behavior, and how does chondroitin sulfate distribution correspond to regional variation in compressive stiffness? Tensile behavior of the disc is better understood than compressive behavior on a biological basis. Under tension, the porcine TMJ disc is much stiffer and stronger in the anteroposterior direction than in the mediolateral direction through the intermediate zone (Detamore and Athanasiou, 2003b). In the mediolateral direction, the TMJ disc is stiffer, stronger, and tougher in the posterior band than in the intermediate zone and anterior band; and stiffer and stronger in turn in the anterior band than in the intermediate zone. Unfortunately, studies of the TMJ disc under compression have been contradictory. For example, one study of porcine discs under compression reported higher stiffnesses in the center of the disc than medially and laterally (Nickel and McLachlan, 1994). However, perhaps due to use of different methodologies, our group has found the porcine disc to be stiffer in the medial region than in the central or lateral regions (Allen and Athanasiou, in press; Kim et al., 2003). Contradictory results have also been seen in the literature for anteroposterior variation of compressive stiffness. In our group, the porcine disc was determined to be stiffer in the posterior band than in the anterior band and in the center of the intermediate zone (Allen and Athanasiou, in press; Kim et al., 2003). In contrast, a study of human TMJ discs found the discs to be stiffer under compression in the intermediate zone than in the anterior and posterior bands (Beek et al., 2001), which has been supported by finite element studies of the human TMJ (Beek et al., 2000; Mizoguchi et al., 1998).

At the heart of these important questions is the tissue engineering community, which strives to produce a native disc equivalent to alleviate problems associated with certain severe and debilitating TMJ disorders by replacing the damaged disc. However, with these questions still lingering, there remains much uncertainty in the validation criteria, and even design criteria, for tissue engineering efforts. The objective of this study was to quantify GAGs in the porcine TMJ disc with ELISAs, and identify their distribution along with the distribution of elastin and collagen types I and II using immunohistochemistry. Scanning electron microscopy (SEM) was used to identify collagen fiber orientation.

2. Results

2.1. Water content

The average wet and dry weights of the four TMJ discs were 2.32±0.87 g and 0.67±0.28 g, respectively. Based on total wet and dry weights, the discs were determined to be 71±2% water by mass. Based on averages of water contents in each region, differences in water content were observed along the anteroposterior and mediolateral axes (Fig. 2), although the superior and inferior layers were nearly identical. Along the mediolateral axis, the medial region (75.3±2.1%) had a higher water content than the central (71.3±3.7%) and lateral (71.3±4.1%) regions ($p<0.0001$). Along the anteroposterior axis, the anterior band (74.5±2.9%) and intermediate zone (73.7±3.1%) had higher water contents than the posterior band (70.1±4.0%; $p<0.0001$). These differences are largely attributed to the lower water contents of the central and lateral regions of the posterior band as shown in Fig. 2. The average water contents of the regions in the superior and inferior layers were 73.0±4.1% and 72.6±3.6%, respectively.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2.2. Quantitative GAG content (ELISAs)

The overall GAG content of the TMJ disc was determined to be 5.3±1.2% DW and 1.5±0.3% WW (including dermatan sulfate proteoglycan). For the dermatan sulfate proteoglycan ELISA, data from one disc were generally below detection limits and were thus discarded. The overall content of each individual GAG is displayed in Fig. 3. There were no significant differences between the superior and inferior layers for any of the GAGs examined (ANOVA: $0.15<p<0.91$); the values displayed in Fig. 3 should be considered representative of both layers. The most abundant GAG in the TMJ disc was chondroitin sulfate, comprising 74% of the total GAG content. There was 4.4 times more chondroitin sulfate than dermatan sulfate proteoglycan, 8.2 times more than keratan sulfate, and 164 times more than hyaluronic acid. Regional variation of the distribution of individual GAGs (with data from inferior and superior layers pooled) is summarized in Table 1 and portrayed in further detail in Fig. 4.

2.2.1. Chondroitin sulfate—Significant differences existed along both the anteroposterior and mediolateral axes for both chondroitin-6-sulfate and chondroitin-4-sulfate. Along the anteroposterior axis, chondroitin-6-sulfate content was 5.5 and 5.3 times higher in the intermediate zone and anterior band, respectively, than in the posterior band ($p<0.0001$). Similarly, the chondroitin-4-sulfate content was 7.7 and 4.5 times higher in the intermediate zone and anterior band, respectively, than in the posterior band ($p<0.0001$). For chondroitin-4-sulfate, there was also a significant difference between the intermediate zone and anterior band, with the intermediate zone having 72% more ($p<0.0001$). Along the mediolateral axis, the medial side of the disc contained more chondroitin sulfate than the lateral side. In particular, the chondroitin-6-sulfate and chondroitin-4-sulfate contents in the medial side were 2.7 and 1.7 times higher than the lateral side, respectively ($p<0.0001$). Chondroitin sulfate in the medial region was also more concentrated than in the central region of the disc, with 82% more chondroitin-6-sulfate ($p<0.0001$) and 34% more chondroitin-4-sulfate (not significant), respectively.

As mentioned above, there was no significant difference between the superior and inferior layers as determined by ANOVA for any of the GAGs. However, there were two locations in the TMJ disc with noteworthy differences in chondroitin sulfate content between layers. In the medial region of the anterior band, the chondroitin-4-sulfate content in the inferior and superior layers was 7.0±3.9% and 1.8±1.2%, respectively. Additionally, in the center of the disc, the chondroitin-6-sulfate content in the inferior and superior layers was 6.2±3.2% and 3.3±0.8%, respectively.

2.2.2. Dermatan sulfate proteoglycan—Regional variation of dermatan sulfate proteoglycan was significant along the anteroposterior axis only. Dermatan sulfate proteoglycan content was highest in the intermediate zone, being 14.4 and 2.5 times higher than in the posterior band ($p<0.0001$) and in the anterior band ($p<0.005$), respectively. In addition, the anterior band contained 5.8 times more dermatan sulfate proteoglycan than the posterior band, although large standard deviations with both groups prevented statistical significance ($p=0.07$).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2.2.3. Keratan sulfate and hyaluronic acid—Like dermatan sulfate proteoglycan, regional differences for keratan sulfate were significant only along the anteroposterior axis, with highest concentrations in the intermediate zone. The keratan sulfate content in the intermediate zone was 94% and 75% higher than in the posterior band and in the anterior band, respectively ($p<0.05$). Values for hyaluronic acid content were so low (0.024% overall) and variable that it was not meaningful to make comparisons among regions. Moreover, these values were not statistically different along any axis as determined by ANOVA ($p>0.4$).

2.3. Extracellular matrix distribution (Immunohistochemistry)

Differences were not observed between the inferior and superior surfaces for any of the eight extracellular matrix components examined. The primary differences revealed immunohistochemically were between the intermediate zone and the posterior and anterior bands. In general, there was little variation mediolaterally, except in the intermediate zone where the medial and lateral edges more closely resembled the anterior and posterior bands.

2.3.1. Collagen and elastin—Collagen I was predominant over collagen II throughout the TMJ disc. The distribution of these collagen types was heterogeneous. The anterior and posterior bands were almost exclusively collagen I (Fig. 5A), although faint collagen II staining was also observed in these regions. However, in the intermediate zone, collagen I staining was discontinuous (Fig. 5B), with regions of intense staining separated by gaps without any staining. Correspondingly, intermittent clusters of collagen II, hundreds of microns across, were observed throughout the intermediate zone (Fig. 5C). Elastin fibers were distributed throughout the TMJ disc, although fibers were more numerous in the posterior band than elsewhere in the tissue (Fig. 6).

2.3.2. GAGs—Chondroitin sulfate and keratan sulfate staining were uniform in the anterior and posterior bands (Fig. 7A,F), and more heterogeneous in the intermediate zone (Fig. 7B,C,E). Dense clusters of keratan sulfate and especially of the chondroitin sulfates appeared throughout the intermediate zone, which were bridged by regions of faint staining or no staining. Serial sections revealed that chondroitin-4-sulfate and chondroitin-6-sulfate stains often filled the respective gaps of the other (Fig. 7B,C). Dermatan sulfate staining was present throughout the disc, although it was most intense in the intermediate zone (Fig. 8A) and faint in the posterior band (Fig. 8B). Hyaluronic acid staining was observed in the medial region of the anterior band, and was faint or absent everywhere else (data not shown).

2.4. Collagen fiber orientation (SEM)

Inside the TMJ disc, collagen fibers had a ring-like structure around the periphery and were oriented anteroposteriorly through the intermediate zone (Fig. 9). Both the anterior and posterior bands were comprised of collagen fibers primarily oriented in a mediolateral direction, along with some branching fibers in the anteroposterior direction. This anteroposterior branching was noticeably more pronounced in the anterior band. The medial and lateral areas contained collagen fibers aligned in an anteroposterior orientation. On the surface of the TMJ disc, a random collagen orientation was observed. The mean collagen

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

fiber diameter observed was 18±9 μm (mean±standard deviation), with a range of 2.9 to 37.4 μm.

3. Discussion

To the best of our knowledge, this is the first study to thoroughly examine the variation of individual GAGs in the TMJ disc. Our results for overall GAG content and regional distribution of individual GAGs appear to agree fairly well with analyses of bovine discs by Nakano and Scott (1989, 1996). However, this is the only study we are aware of to investigate three dimensional, three-axis variation of the content of individual GAGs in the TMJ disc.

With the information provided in this study, we can now directly address the questions posed earlier. The first question was in regard to the presence of collagen II. The current study provides supporting evidence for the presence of type II collagen (Fig. 5). While collagen II is present throughout the TMJ disc in trace quantities, the intermediate zone presents a pattern of discontinuous regions of collagen I separated by collagen II, perhaps in a manner somewhat similar to that observed by Mills et al. (1994). It can thus be concluded that collagen II does indeed exist in the TMJ disc and, while present only in minute quantities, is more concentrated in the intermediate zone than elsewhere in the disc.

Another question addressed by this study is how elastin fibers are distributed in the disc. The current study (Fig. 6) supports a previous study of porcine discs (Christensen, 1975), as it is also determined here that elastin fibers are most dense in the posterior band. This may suggest a difference in elastin distribution between porcine and human TMJ discs. A smaller relative number of elastin fibers in the intermediate zone is not observed here as was seen in previous reports (Christensen, 1975; Gross et al., 1999; O'Dell et al., 1990). In addition, although it has been reported that there is more elastin in the superior layer than the inferior layer by a 60/40 ratio (Gross et al., 1999), a difference between layers is not observed immunohistochemically in the current study.

This study also provides answers to a number of questions with regard to GAGs in the TMJ disc by identifying the overall GAG content, the primary GAGs, and their distribution. The results show the overall GAG content (including the mass of the dermatan sulfate proteoglycan, and not including heparan sulfate or heparin) to be 5.3±1.2% of the dry weight. This value falls within the previously reported range of 0.5% to 10% in human, porcine, bovine, and rat TMJ discs (Almarza et al., accepted for publication; Axelsson et al., 1992; Nakano and Scott, 1989, 1996; Okazaki et al., 1996; Sindelar et al., 2000), and is most similar to the 5% GAG content reported in bovine discs (Nakano and Scott, 1989) and 3.24% glucuronic acid content reported in rat discs (Okazaki et al., 1996). It should be noted that widely varying methodologies have been used to assess total GAG content, which means that exactly the same thing is not being measured in all of these studies. Therefore, one should use caution in making direct comparisons of quantitative GAG contents from these studies. Methodologies include histological staining and absorbance (Sindelar et al., 2000), ion-exchange chromatography (Nakano and Scott, 1989), electrophoresis (Nakano and Scott, 1996; Okazaki et al., 1996), HPLC (Axelsson et al., 1992), and ELISA (Nakano

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and Scott, 1996). In addition, uronic acid content (either glucuronic or iduronic acid) has been measured (Nakano and Scott, 1996; Okazaki et al., 1996). Glucuronic acid is present in chondroitin sulfate and hyaluronic acid; whereas iduronic acid is present in dermatan sulfate, heparin and heparan sulfate; and neither are present in keratan sulfate. These studies and their methodologies are discussed at length and tabulated in a recent review (Detamore and Athanasiou, 2003a).

A recent similar regional analysis in our group of porcine discs found the overall GAG content to be 0.96±0.39% of the dry weight (Almarza et al., accepted for publication). Differences in observed values can be attributed to differing methodologies. Although tissue digestion procedures were identical, the previous study utilized a dimethylmethylene blue dye-binding assay to assess overall sulfated GAG content, whereas the current study employed an ELISA approach to quantify individual GAGs, including dermatan sulfate proteoglycan, and summing their contents to determine a total. Despite the discrepancy in total GAG content, similar trends were observed between these two studies. In our prior study of total GAG content, anteroposteriorly the intermediate zone contained more GAGs than the anterior band, which in turn contained more than the posterior band (Almarza et al., accepted for publication). This same trend is observed in the current study to an extent with chondroitin sulfate, keratan sulfate, and dermatan sulfate (Table 1). Mediolaterally, our analysis of chondroitin sulfate appears to agree with our prior assessment of overall GAG content, with higher concentrations in the medial region.

Previous reports of regional variation of GAG content have been inconsistent (Kuwabara et al., 2002; Mizoguchi et al., 1998; Nakano and Scott, 1996; Sindelar et al., 2000). The results of the current study, with GAG contents generally being highest in the intermediate zone, would best correspond to the study of bovine TMJ discs (Nakano and Scott, 1996), where GAG contents were higher in the center of the disc compared to the periphery. In addition, the literature is contradictory as to whether the most prevalent GAG in the TMJ disc is chondroitin sulfate or dermatan sulfate (Axelsson et al., 1992; Nakano and Scott, 1989, 1996; Okazaki et al., 1996). The current study supports the studies of bovine discs (Nakano and Scott, 1989, 1996), with chondroitin sulfate representing 74% of the total GAG content.

The final question that the study addresses is whether hyaluronic acid and keratan sulfate comprise a significant portion of the GAG population. Keratan sulfate in the current study is determined to be 0.83±0.64% of the dry weight in the intermediate zone, which correlates well to the previously reported value of 0.95±0.33% in the center of the bovine disc (Nakano and Scott, 1996). We conclude that keratan sulfate is indeed present, although it is a minor constituent, and exhibits anteroposterior concentration variations with highest amounts in the intermediate zone. The values for hyaluronic acid may have been slightly lower due to competitive binding of sample proteins to the well plates. However, the current findings place hyaluronic acid content significantly lower than keratan sulfate content, supporting a prior study that found higher amounts of keratan sulfate than hyaluronic acid (Nakano and Scott, 1996).

Although it is known that the TMJ disc is different from hyaline cartilage and knee meniscus (Almarza and Athanasiou, 2004), the TMJ disc has been often confused with these fellow

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

cartilaginous tissues. This study paints a distinct portrait of the TMJ disc that can be compared side-by-side to these other more studied and more well-understood tissues. With regard to collagen types, hyaline cartilage is almost exclusively collagen II, and the knee meniscus collagen is over 80% type I (Cheung, 1987; Eyre and Wu, 1983), with type II collagen occurring primarily in the inner region. From the current study, we can state that the collagen of the TMJ disc is almost exclusively type I with traces of collagen type II. The overall GAG content of hyaline cartilage is generally in the range of 25% to 30% of the dry weight, whereas the total GAG content in the knee meniscus falls in the range of 2% to 3% of the tissue dry weight (Almarza and Athanasiou, 2004). In comparison, the current study shows the overall GAG content of the TMJ disc to lie at about 5%. Moreover, it shows that chondroitin sulfate is the primary GAG of the TMJ disc, likely followed by dermatan sulfate and then keratan sulfate. In hyaline cartilage, the primary GAGs are chondroitin sulfate and keratan sulfate, whereas in the knee meniscus, chondroitin sulfate is the most abundant GAG, followed by dermatan sulfate (Almarza and Athanasiou, 2004). With regard to water content, water makes up 75–80% of the wet weight of hyaline cartilage (Maroudas, 1979), whereas the meniscus is about 72% water (Herwig et al., 1984). In comparison, the present study found the TMJ disc to be 71±2% water. Therefore, in terms of extracellular matrix and water content, the TMJ disc appears to be more similar to the knee meniscus. However, differences in cell types, and in matrix organization and resulting mechanical properties between the knee meniscus and the TMJ disc result in these tissues being much more distinct from one another than examination of matrix content alone may suggest.

In addition to comparing the TMJ disc to other tissues, we can now better understand how organization of its extracellular matrix is manifested in its observed biomechanical behavior, namely under tension and compression. The anisotropic and heterogeneous orientation of collagen fibers, as observed with SEM (Fig. 9), results in the anisotropic and heterogeneous behavior of the TMJ disc under tension (Detamore and Athanasiou, 2003b). In addition, chondroitin sulfate proteoglycans contribute to compressive stiffness, which is attributed to the ability of these highly anionic macromolecules to trap water, which can be considered incompressible (Carvalho et al., 1995; Detamore and Athanasiou, 2003c). This link is clearly demonstrated in the strong correlation between water content distribution (Fig. 2) and chondroitin sulfate distribution (Table 1). In the current study, chondroitin sulfate was most concentrated in the intermediate zone and to a lesser degree the anterior band, which would better correspond to compression studies of the human TMJ disc, which found the intermediate zone to be the stiffest region. Perhaps the differences lie in the distinction between relaxed and instantaneous moduli. Moduli obtained for human discs were instantaneous, whereas those for porcine discs were relaxed moduli. Since the posterior band already starts with less water, perhaps the instantaneous response is governed by chondroitin sulfate content, which is highest in the intermediate zone. Hereafter, perhaps after prolonged stresses, there is less water to be dissipated from the posterior band and thus less prolonged compliance, a phenomenon that could possibly be attributed to the highly organized collagen fibers in the posterior band, which actually give rise to the highest tensile stiffness, strength, and toughness seen in the TMJ disc. However, this is merely conjecture, made in an attempt to understand deviation from the traditional relationship between chondroitin sulfate content and compressive stiffness that occurs along the anteroposterior axis in the TMJ disc.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In addition to correlating matrix content to observed mechanical properties, it may be prudent to also consider the cells of the TMJ disc. The cells of the TMJ disc are approximately 70% fibroblasts and 30% fibrochondrocytes, with fibrochondrocytes being more prevalent in the intermediate zone than in the anterior and posterior bands (Detamore et al., submitted for publication). Fibrochondrocytes of the TMJ disc resemble chondrocytes of hyaline cartilage, although there are differences in pericellular matrix and organelle content (Detamore et al., submitted for publication). However, it may be inferred that production of matrix components associated with hyaline cartilage (i.e., collagen II, chondroitin sulfate/keratan sulfate proteoglycans) would be better attributed to the fibrochondrocytes than to the fibroblasts. Indeed, there is a remarkable correlation, in that significantly higher amounts of chondroitin sulfate and keratan sulfate (Table 1), and of collagen II (Fig. 5), are observed in the intermediate zone of the disc than in the anterior and posterior bands. To the best of our knowledge, this is the first time that this correlation has been identified.

At this point in time, we can begin to decipher the overall composition of the TMJ disc. In a previous study in our group, collagen was determined to account for 69±8% of the dry weight of the disc as calculated in the same manner as overall GAG content here (Almarza et al., accepted for publication). The overall GAG content in the current study is determined to be 5.3±1.2% of the dry weight. This leaves 25% of the overall content of the dry weight of the TMJ disc unaccounted for by GAGs and collagen. However, there is a cellular component to the dry weight, which can be estimated to be roughly 10% after considering that the TMJ disc contains 1.4 mg DNA/g (dry weight) and 7.7 pg DNA/cell (Almarza et al., accepted for publication) and calculating cell volume assuming a spherical cell with a 10 Am diameter and a density of 1.1 g/mL. In addition, dry weight can be assigned to elastin, which has been reported to account for between 2% and 7% of the dry weight of the disc (Keith, 1979; O'Dell et al., 1990). It should be noted that the GAG content determined in this study includes the entire dermatan sulfate proteoglycan mass, but does not include the protein component of the chondroitin sulfate/keratan sulfate proteoglycans. Finally, there are certainly a multitude of other constituents comprising a smaller percent of the dry weight of the TMJ disc, including fibronectin (Milam et al., 1991; Mills et al., 1994) and tenascin (Milam et al., 1991), amongst others.

Now that our group has characterized the TMJ disc at the ultrastructural, cellular, extracellular matrix (structural), and biomechanical (functional) levels, there is one pervading universal theme in the heterogeneity of the disc: the primary regional differences in the disc exist between the intermediate zone and the anterior and posterior bands. In comparison to the anterior and posterior bands, the intermediate zone has a higher proportion of fibrochondrocytes (Detamore et al., submitted for publication), higher quantities of total collagen (Almarza et al., accepted for publication) and of type II collagen (Fig. 5), higher quantities of chondroitin sulfate, keratan sulfate, and dermatan sulfate proteoglycan (Table 1), a higher quantity of total GAG (Almarza et al., accepted for publication), and is over an order of magnitude softer and weaker under mediolateral tension (Detamore and Athanasiou, 2003b). To a lesser extent, the medial region appears to differ from the lateral region. In comparison to the lateral region of the disc, the medial region is stiffer under compression (Allen and Athanasiou, in press; Kim et al., 2003) and under

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

anteroposterior tension (Detamore and Athanasiou, 2003b), and has higher levels of chondroitin sulfate (Table 1) and total GAG (Almarza et al., accepted for publication).

As the field of tissue engineering for the TMJ disc matures, researchers may attempt to emulate the efforts of the hyaline cartilage tissue engineering community, where efforts are presently made to regenerate the zonal architecture of articular cartilage by means such as culturing separate populations of cells. If those who endeavor to engineer the TMJ disc wish to follow an analogous approach, the first basic step would be to differentiate between the intermediate zone and the two bands. Fifty years after the first investigation of the TMJ disc that coined the terms anterior band, posterior band and intermediate zone (Rees, 1954), we now have a much deeper understanding of the nature of the differences among these regions that will guide us in our efforts to reproduce this regional variation in engineering a native TMJ disc equivalent.

4. Materials and methods

4.1. Materials

4.1.1. Standards—Bovine decorin was obtained from Sigma-Aldrich (St. Louis, MO). Porcine hyaluronic acid and chondroitin-4-sulfate, shark chondroitin-6-sulfate and bovine keratan sulfate were obtained from Carbomer (Westborough, MA).

4.1.2. Primary antibodies—The mouse monoclonal IgG anti-chondroitin-6-sulfate, mouse monoclonal IgG anti-chondroitin-4-sulfate, and mouse monoclonal IgG anti-keratan sulfate antibodies (Poole et al., 1991) were obtained from Chemicon International (Temecula, CA). The mouse monoclonal IgM anti-chondroitin sulfate/anti-dermatan sulfate antibody, PG-4, was a generous gift from the group of Arnold Caplan (Sorrell et al., 1999). Digestion of a sample with chondroitinase AC prior to using PG-4 allows for exclusive recognition of dermatan sulfate with immunohistochemistry. It was determined in preliminary experiments that 3 h of chondroitinase AC digestion was sufficient to eliminate chondroitin sulfate detection. PG-4 was not used to detect dermatan sulfate by ELISA, as the chondroitinase AC digestion step cleaves dermatan sulfate chains at chondroitin sulfate impurities. Since we were unable to locate antibodies for the dermatan sulfate glycosaminoglycan itself, dermatan sulfate proteoglycan was analyzed instead for ELISAs. The mouse monoclonal IgG anti-dermatan sulfate proteoglycan antibody was obtained from Calbiochem (San Diego, CA). The mouse monoclonal IgG anti-elastin monoclonal antibody was obtained from Vector Laboratories (Burlingame, CA), and the sheep polyclonal IgG anti-hyaluronic acid antibody was obtained from Biogenesis (Kingston, NH). The mouse monoclonal IgG anti-collagen I and mouse monoclonal IgG anti-collagen II antibodies were obtained from Accurate Chemical and Scientific (Westbury, NY) and Chondrex, LLC (Redmond, WA), respectively.

4.1.3. Secondary antibodies and visualization—For immunohistochemistry, avidin–biotin complex kits were obtained from Vector Laboratories (Burlingame, CA). The kits consisted of a biotinylated secondary antibody (either horse anti-mouse IgG, rabbit anti-sheep IgG, or goat anti-mouse IgM), the corresponding host animal serum, and an avidin–biotinylated enzyme complex. Samples for immunohistochemistry were visualized using

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3,3′-diaminobenzidine (DAB). For ELISAs, peroxidase-conjugated goat anti-mouse IgG and donkey anti-sheep IgG secondary antibodies were purchased from Chemicon International. The visualizing agent for ELISA was 3,3′,5,5′-tetramethyl-benzidine (TMB).

4.1.4. Tissue samples—Hog heads (P.I.C. Genetic Breed) were obtained from a local abattoir (Fisher Ham and Meat; Spring, TX). All hogs were females approximately 6 months of age and in the weight range of 150–180 lb (70–80 kg).

4.2. Methods

4.2.1. Specimen preparation—Hog heads were acquired immediately after death, and their TMJ discs were removed the same day. The discs were dissected free of attachment tissues, and washed in 0.01 M phosphate-buffered saline (PBS-0.138 M sodium chloride, 0.0027 M potassium chloride) to remove synovial fluid. The gross morphology of the discs was inspected, and all appeared to be normal.

For quantitative ELISAs, four left discs were each cut into 18 regions with a scalpel (see Fig. 1B for reference). First, discs were cut mediolaterally into three regions to separate the anterior band, intermediate zone, and posterior band. Next, each strip was cut anteroposteriorly approximately into thirds. Finally, each region was cut approximately in half, separating the superior portion from the inferior portion. Each individual sample was wrapped in PBS-saturated gauze and stored at −20 °C until ready for ELISA examination.

For topographical immunohistochemistry, two right discs from different heads were cut into nine regions mediolaterally and anteroposteriorly in the same manner as described for ELISA specimens. To maintain specimen orientation, the superior and anterior surfaces of each region were marked with black and purple India ink, respectively. These portions were stored in PBS-saturated gauze at −20 °C prior to sectioning. Serial 10 μm frozen sections were taken from each of the nine regions at two layers along the supero-inferior axis: near the superior surface, and near the inferior surface. In this manner, 18 regions from each disc could be examined for each immunohistochemical assay. Sections were mounted on slides, fixed in chilled acetone for 20 min and then stored at −20 °C until further use.

For scanning electron microscopy (SEM), a whole TMJ disc was fixed in formalin for 24 h. Samples were obtained from five representative areas of the TMJ disc: the anterior band and posterior band, and the medial, central and lateral regions of the intermediate zone. These samples were then split by hand through the middle to expose inner collagen fibers in a manner such that sample orientation could be traced. The samples were then placed back in formalin for another 24 h before subsequent SEM preparation.

4.2.2. Enzyme-linked immunosorbent assay (ELISA)—Protocol parameters for ELISAs are listed in Table 2. Each specimen was removed from gauze and rehydrated for 4 h in PBS, gently blotted to remove excess moisture, and weighed to obtain a wet weight. All specimens were then lyophilized for 48 h and weighed again to measure dry weights. Specimens were then solubilized in 1.5 mL of 125 mg/mL papain overnight at 60 °C. All samples, standards and antibodies were diluted using PBS. Washes between ELISA steps were performed using PBS with 0.05% Tween-20.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Inhibition ELISAs were used to quantify chondroitin-4-sulfate, chondroitin-6-sulfate, keratan sulfate, and dermatan sulfate proteoglycan. For the dermatan sulfate proteoglycan inhibition ELISAs, high-protein-binding well plates were coated with decorin by overnight incubation. The following day, the plates were blocked by incubating with 5% bovine serum albumin in PBS with 0.05% Tween-20 and 0.02% sodium azide for 2 h. During this time, 125 μL of primary antibody was added to 125 μL of each sample and standard and incubated for 1 h. The samples and standards with primary antibody were added to the well plates for 1 h, followed by a secondary antibody for 1 h. TMB was added and given 10–15 min to develop color, then the reaction was stopped with 1 M phosphoric acid and the plates were read at 450 nm.

For the chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate inhibition ELISAs, the plates were coated with aggrecan by overnight incubation. The next day, 0.1 U/mL chondroitinase ABC (Seikagaku America; East Falmouth, MA) was added to the plates for 2 h, followed by a 2 h blocking step as described above. During these steps, samples and standards were incubated with chondroitinase ABC (0.1 U/mL for chondroitin-4-sulfate and chondroitin-6-sulfate, 0.02 U/mL for keratan sulfate) for 2 h, followed by a 1 h incubation of samples and standards with the respective primary antibody. Hereafter, the procedure followed as for the dermatan sulfate proteoglycan ELISA, by adding sample/standard/primary antibody to the plates and following with a secondary antibody and TMB.

Indirect ELISAs were used to quantify hyaluronic acid. The plates were coated with standards and samples overnight. Following the blocking step, performed as described above, the plates were exposed to the primary antibody for 1 h, the secondary antibody for 1 h, then visualized with TMB as described above.

For statistical analysis, a three-factor ANOVA was performed, where the three factors were the three axes along which the disc was divided. The Fisher's protected least significant difference (PLSD) post hoc test was employed when significant differences were found with ANOVAs. All regional analyses of GAG content were performed with dry weight data only. Values for the content of specific GAGs for each region were calculated by averaging mass concentrations of individual specimens (where the objective was to examine variation in concentrations). In contrast, the content of each GAG type in the disc overall was calculated by first summing the total mass of each respective GAG for an entire disc and then dividing by the total dry or wet weight of that disc, to give an actual mass percent of each GAG in the original intact TMJ disc.

4.2.3. Immunohistochemistry—Parameters for immunohistochemistry protocols are listed in Table 3. Immunostaining was performed using a Biogenex i6000 (San Ramon, CA) autostainer. Slides were placed in the autostainer and rehydrated for 5 min in PBS. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide in methanol for 30 min, followed by an appropriate tissue digestion, if necessary (see Table 3). Specimens were blocked with serum from the secondary antibody host for 20 min (horse and rabbit serum: 3%; goat serum: 1.5%) and followed by incubation with the primary antibody for 60 min. The specimens were then incubated with a secondary antibody in host serum (same concentration as block) for 30 min, followed by the avidin–biotinylated enzyme complex for

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

30 min and then DAB for 4 min. The anti-mouse IgG antibody solution contained 2% porcine serum to prevent non-specific binding. Slides were removed from the autostainer, counterstained with hematoxylin, dehydrated in graded ethanol and mounted.

4.2.4. Scanning electron microscopy (SEM)—Following formalin fixation, the specimens were covered with 2 N sodium hydroxide for 7 days to expose the collagen fibers. To clear debris, the samples were placed in distilled water for approximately 2 weeks. Samples were placed under 2% tannic acid for 5 h and returned to distilled water for another 24 h. Samples were then post-fixed with osmium tetroxide for 2 h, dehydrated with graded ethanol, and critical point dried with CO_2. Samples were sputter coated with gold and examined with a Philips XL-30 ESEM-FEG microscope under magnifications up to 25,000×. Collagen fiber diameters were measured for 18 different fibers.

Acknowledgments

We gratefully acknowledge funding from National Institute of Dental and Craniofacial Research, grants #R01 DE015038-01A2 and #T35 DE07252-110, from the Whitaker Foundation, and from the Nettie S. Autrey Memorial Fellowship. In addition, we would like to thank Ms. Julie Horng for her contribution to the initial development of ELISA protocols, Dr. Zahid Lalani of the University of Texas Health Science Center-Houston Dental School for initial guidance with immunohistochemistry protocols, Mr. Mark Sweigart for guidance with the SEM protocols, and Drs. David Carrino and Arnold Caplan for their generous contribution of the PG-4 antibody.

References

Allen KD, Athanasiou KA. Viscoelastic characterization of the porcine temporomandibular joint disc under unconfined compression. J. Biomech. 2005 in press.

Almarza AJ, Athanasiou KA. Design characteristics for the tissue engineering of cartilaginous tissues. Ann. Biomed. Eng. 2004; 32:2–17. [PubMed: 14964717]

Almarza AJ, Bean AC, Baggett LS, Athanasiou KA. Biochemical content and distribution in the porcine temporomandibular joint disc. J. Oral Maxillofac. Surg. 2005 Accepted for publication.

Axelsson S, Holmlund A, Hjerpe A. Glycosaminoglycans in normal and osteoarthrotic human temporomandibular joint disks. Acta Odontol. Scand. 1992; 50:113–119. [PubMed: 1604965]

Beek M, Koolstra JH, van Ruijven LJ, van Eijden TM. Three-dimensional finite element analysis of the human temporomandibular joint disc. J. Biomech. 2000; 33:307–316. [PubMed: 10673114]

Beek M, Aarnts MP, Koolstra JH, Feilzer AJ, van Eijden TM. Dynamic properties of the human temporomandibular joint disc. J. Dent. Res. 2001; 80:876–880. [PubMed: 11379888]

Carvalho RS, Yen EH, Suga DM. Glycosaminoglycan synthesis in the rat articular disk in response to mechanical stress. Am. J. Orthod. Dentofac. Orthop. 1995; 107:401–410.

Cheung HS. Distribution of type I, II, III and V in the pepsin solubilized collagens in bovine menisci. Connect. Tissue Res. 1987; 16:343–356. [PubMed: 3132349]

Christensen LV. Elastic tissue in the temporomandibular disc of miniature swine. J. Oral Rehabil. 1975; 2:373–377. [PubMed: 23150914]

Detamore MS, Athanasiou KA. Motivation, characterization, and strategy for tissue engineering the temporomandibular joint disc. Tissue Eng. 2003a; 9:1065–1087. [PubMed: 14670096]

Detamore MS, Athanasiou KA. Tensile properties of the porcine temporomandibular joint disc. J. Biomech. Eng. 2003b; 125:558–565. [PubMed: 12968581]

Detamore MS, Athanasiou KA. Structure and function of the temporomandibular joint disc: implications for tissue engineering. J. Oral Maxillofac. Surg. 2003c; 61:494–506. [PubMed: 12684970]

Detamore MS, Hegde JN, Wagle RR, Almarza AJ, Montufar-Solis D, Duke PJ, Athanasiou KA. Cell type and distribution in the porcine temporomandibular joint disc. J. Oral Maxillofac. Surg. 2005 Submitted for publication.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Eyre DR, Wu JJ. Collagen of fibrocartilage: a distinctive molecular phenotype in bovine meniscus. FEBS Lett. 1983; 158:265–270. [PubMed: 6688225]

Fujita S, Hoshino K. Histochemical and immunohistochemical studies on the articular disk of the temporomandibular joint in rats. Acta Anat. 1989; 134:26–30. [PubMed: 2718712]

Gross A, Bumann A, Hoffmeister B. Elastic fibers in the human temporo-mandibular joint disc. Int. J. Oral Maxillofac. Surg. 1999; 28:464–468. [PubMed: 10609752]

Herwig J, Egner E, Buddecke E. Chemical changes of human knee joint menisci in various stages of degeneration. Ann. Rheum. Dis. 1984; 43:635–640. [PubMed: 6548109]

Keith DA. Elastin in the bovine mandibular joint. Arch. Oral Biol. 1979; 24:211–215. [PubMed: 289359]

Kim KW, Wong ME, Helfrick JF, Thomas JB, Athanasiou KA. Biomechanical tissue characterization of the superior joint space of the porcine temporomandibular joint. Ann. Biomed. Eng. 2003; 31:924–930. [PubMed: 12918907]

Kondoh T, Hamada Y, Iino M, Takahashi T, Kikuchi T, Fujikawa K, Seto K. Regional differences of type II collagen synthesis in the human temporomandibular joint disc: immunolocalization study of carboxy-terminal type II procollagen peptide (chondrocalcin). Arch. Oral Biol. 2003; 48:621–625. [PubMed: 12887996]

Kuwabara M, Takuma T, Scott PG, Dodd CM, Mizoguchi I. Biochemical and immunohistochemical studies of the protein expression and localization of decorin and biglycan in the temporomandibular joint disc of growing rats. Arch. Oral Biol. 2002; 47:473–480. [PubMed: 12102764]

Landesberg R, Takeuchi E, Puzas JE. Cellular, biochemical and molecular characterization of the bovine temporomandibular joint disc. Arch. Oral Biol. 1996; 41:761–767. [PubMed: 9022913]

Maroudas, A. Physicochemical properties of articular cartilage. In: Freeman, MAR., editor. Adult articular cartilage. Pitman Medical Kent; UK: 1979. p. 215-290.

Milam SB, Klebe RJ, Triplett RG, Herbert D. Characterization of the extracellular matrix of the primate temporomandibular joint. J. Oral Maxillofac. Surg. 1991; 49:381–391. [PubMed: 1706426]

Mills DK, Fiandaca DJ, Scapino RP. Morphologic, microscopic, and immunohistochemical investigations into the function of the primate TMJ disc. J. Orofac. Pain. 1994; 8:136–154. [PubMed: 7920350]

Mizoguchi I, Scott PG, Dodd CM, Rahemtulla F, Sasano Y, Kuwabara M, Satoh S, Saitoh S, Hatakeyama Y, Kagayama M, Mitani H. An immunohistochemical study of the localization of biglycan, decorin and large chondroitin-sulphate proteoglycan in adult rat temporomandibular joint disc. Arch. Oral Biol. 1998; 43:889–898. [PubMed: 9821512]

Nakano T, Scott PG. A quantitative chemical study of glycosaminoglycans in the articular disc of the bovine temporomandibular joint. Arch. Oral Biol. 1989; 34:749–757. [PubMed: 2516441]

Nakano T, Scott PG. Changes in the chemical composition of the bovine temporomandibular joint disc with age. Arch. Oral Biol. 1996; 41:845–853. [PubMed: 9022922]

Nickel JC, McLachlan KR. In vitro measurement of the stress-distribution properties of the pig temporomandibular joint disc. Arch. Oral Biol. 1994; 39:439–448. [PubMed: 8060268]

O'Dell NL, Sharawy M, Pennington CB, Marlow RK. Distribution of putative elastic fibers in rabbit temporomandibular joint tissues. Acta Anat. 1989; 135:239–244. [PubMed: 2782019]

O'Dell NL, Starcher BC, Wilson JT, Pennington CB, Jones GA. Morphological and biochemical evidence for elastic fibres in the Syrian hamster temporomandibular joint disc. Arch. Oral Biol. 1990; 35:807–811. [PubMed: 2264798]

Okazaki J, Kamada A, Higuchi Y, Kanabayashi T, Sakaki T, Gonda Y. Age changes in the rat temporomandibular joint articular disc: a biochemical study on glycosaminoglycan content. J. Oral Rehabil. 1996; 23:536–540. [PubMed: 8866266]

Poole CA, Glant TT, Schofield JR. Chondrons from articular cartilage: (IV). Immunolocalization of proteoglycan epitopes in isolated canine tibial chondrons. J. Histochem. Cytochem. 1991; 39:1175–1187. [PubMed: 1717545]

Rees LA. The structure and function of the mandibular joint. Br. Dent. J. 1954; 96:125–133.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Sindelar BJ, Evanko SP, Alonzo T, Herring SW, Wight T. Effects of intraoral splint wear on proteoglycans in the temporomandibular joint disc. Arch. Biochem. Biophys. 2000; 379:64–70. [PubMed: 10864442]

Sorrell JM, Carrino DA, Baber MA, Asselineau D, Caplan AI. A monoclonal antibody which recognizes a glycosaminoglycan epitope in both dermatan sulfate and chondroitin sulfate proteoglycans of human skin. Histochem. J. 1999; 31:549–558. [PubMed: 10507462]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

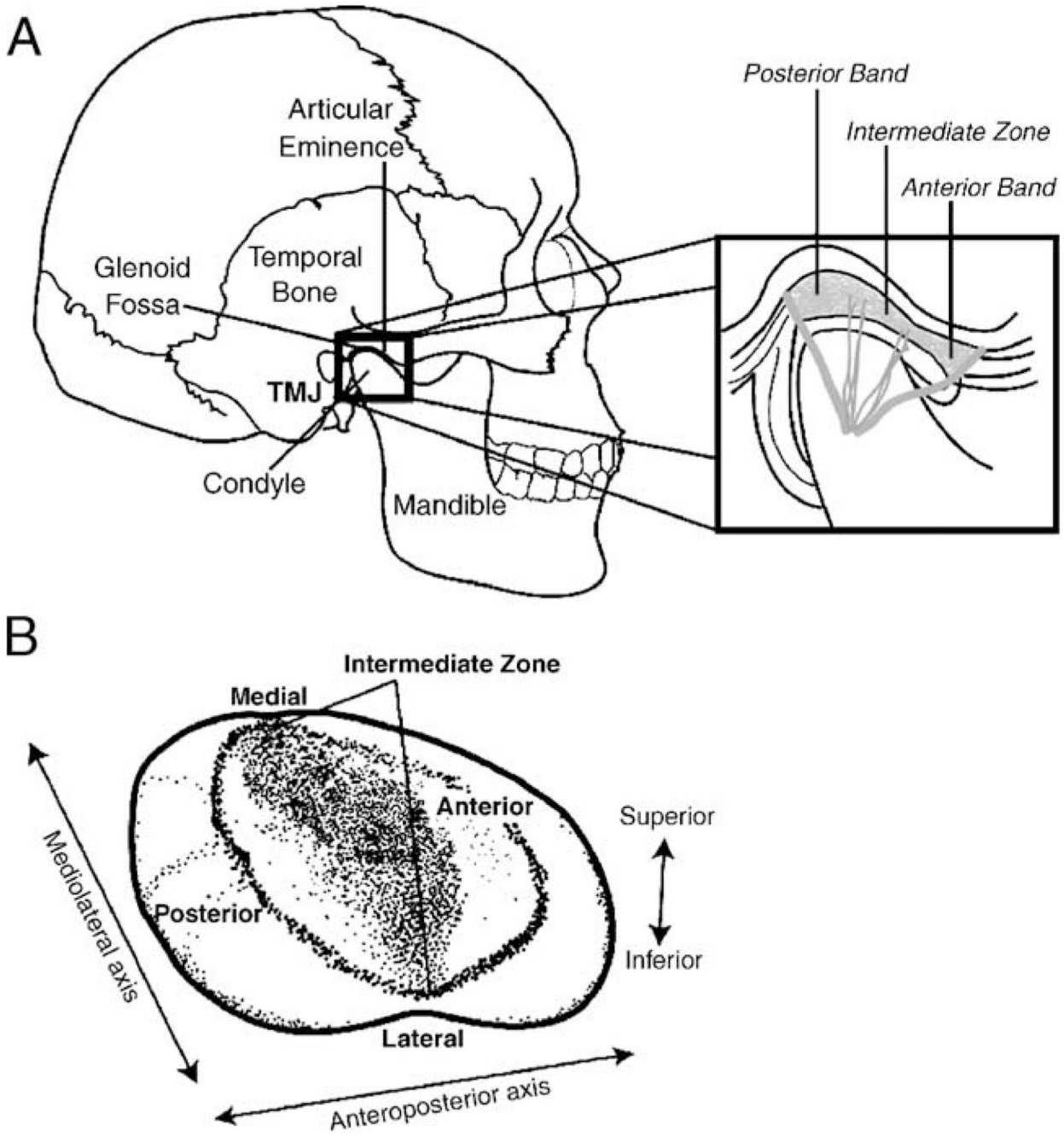

Fig. 1.

Schematic of the TMJ and its disc. (A) Exploded view of TMJ, showing the disc from a lateral view as it is situated in the joint with its attachments. (B) Superior 3D view of the TMJ disc, highlighting its regions and three axes.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

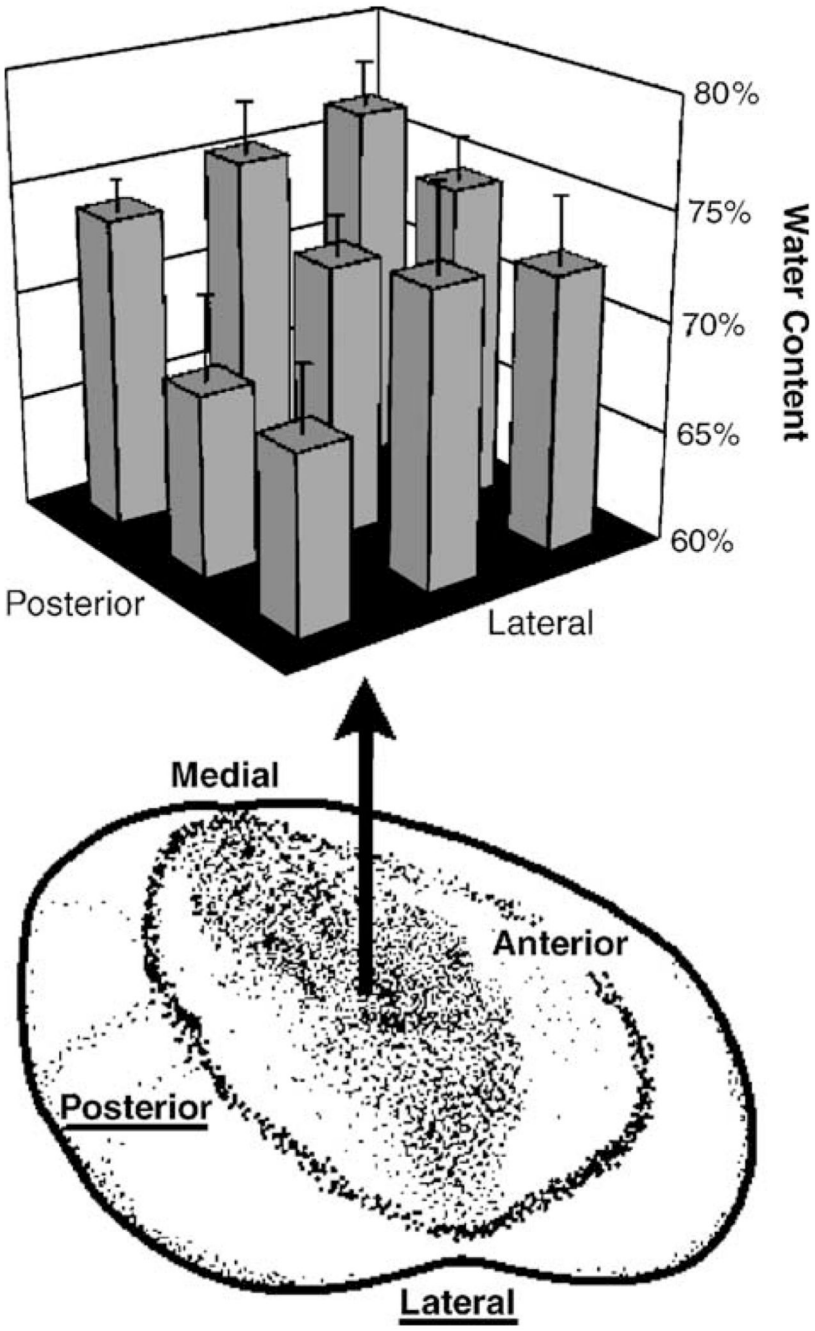

Fig. 2.

Water content of the TMJ disc. A schematic of the TMJ disc serves as a reference point for locations displayed on the bar graph. Data for inferior and superior surfaces pooled, resulting in eight total observations from four discs for each of the nine displayed regions. Errors are standard deviations.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

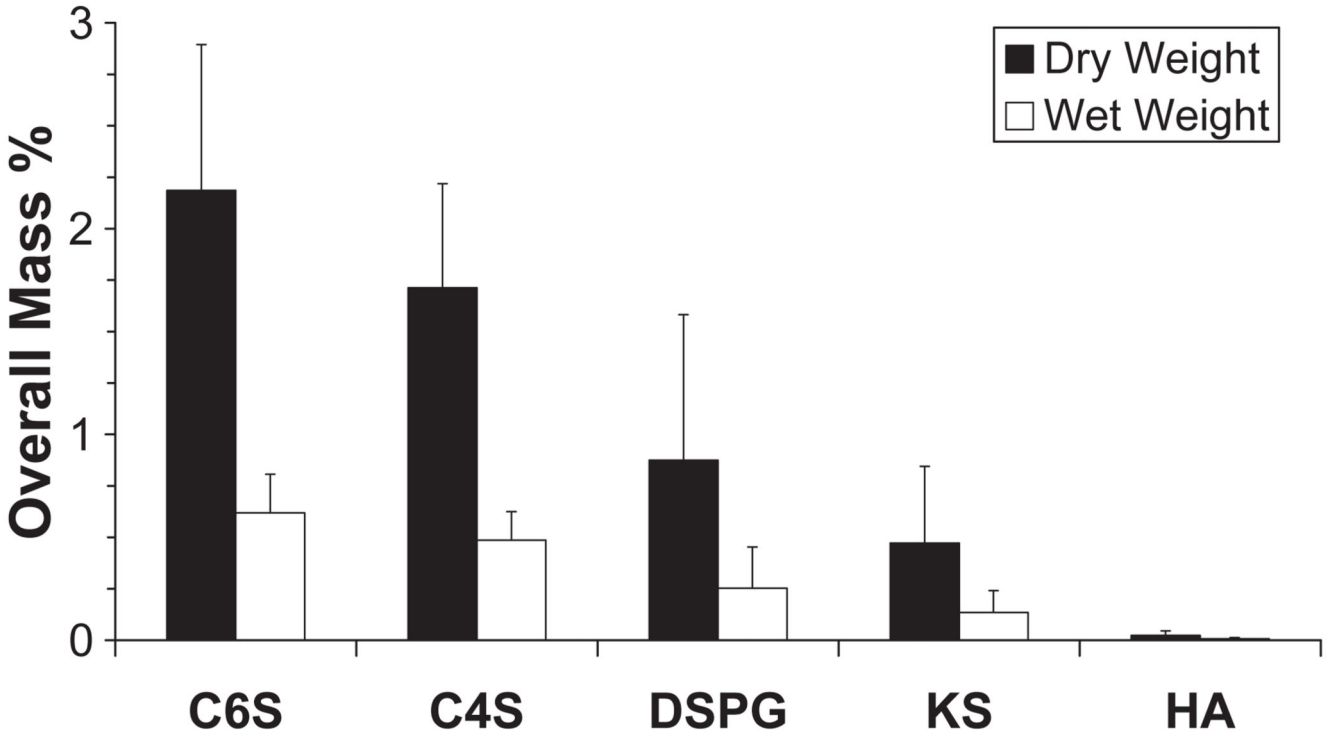

Fig. 3.

Overall mass percent of each GAG in the TMJ disc. Values represent total mass of each GAG divided by the total mass of the disc for each disc, rather than an average of mass percents of GAGs for the 18 different regions. Errors are standard deviations. C4S—chondroitin-4-sulfate, C6S— chondroitin-6-sulfate, DSPG—dermatan sulfate proteoglycan, KS—keratan sulfate, HA—hyaluronic acid.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

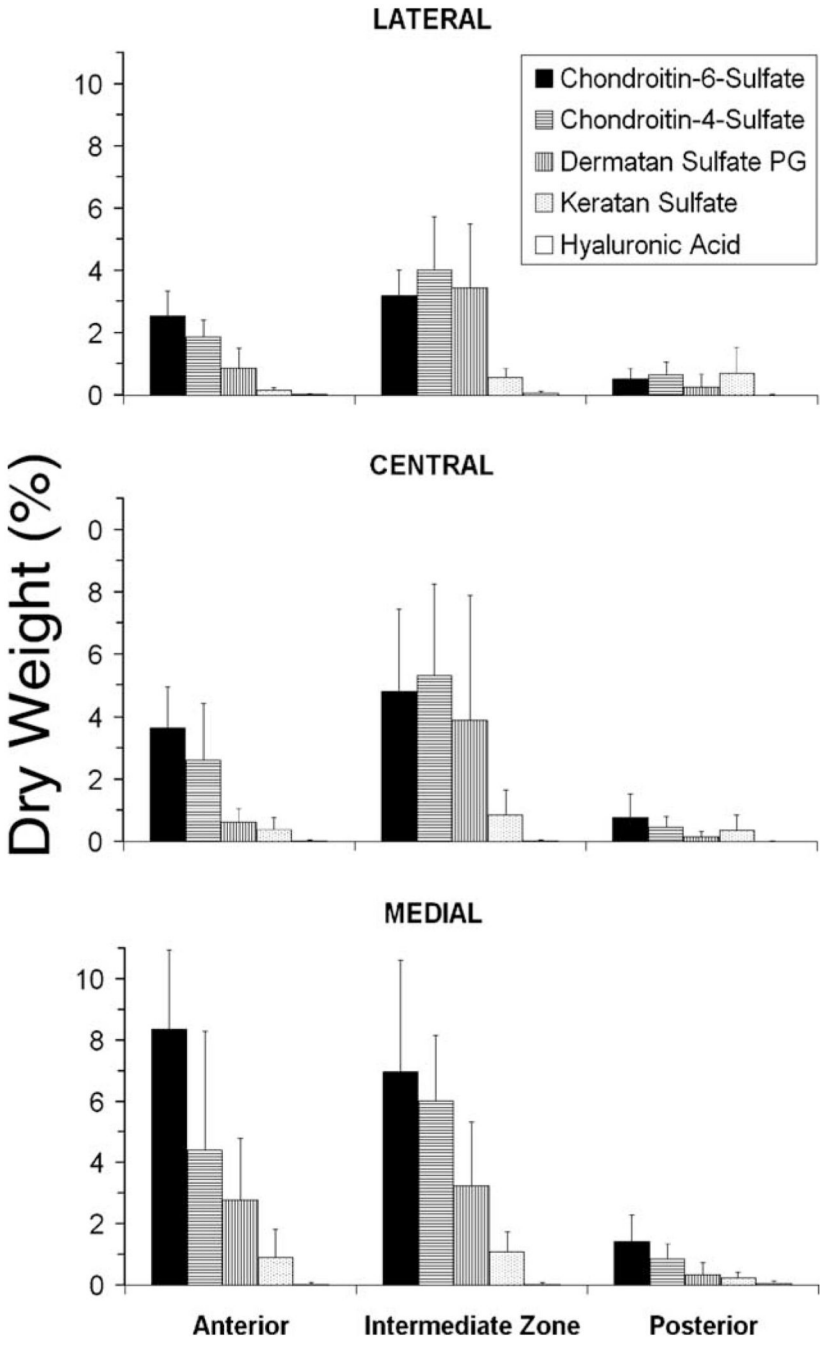

Fig. 4.

Regional variations of individual GAGs in the TMJ disc. Data for inferior and superior layers combined, resulting in eight total observations from four discs for each of the nine displayed regions. Values represent an average of GAG mass percents of the different samples. Note the progressive increase in chondroitin and keratan sulfate in the anterior band and intermediate zone from the lateral to medial region. Errors are standard deviations. Abbreviations as for Fig. 3.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

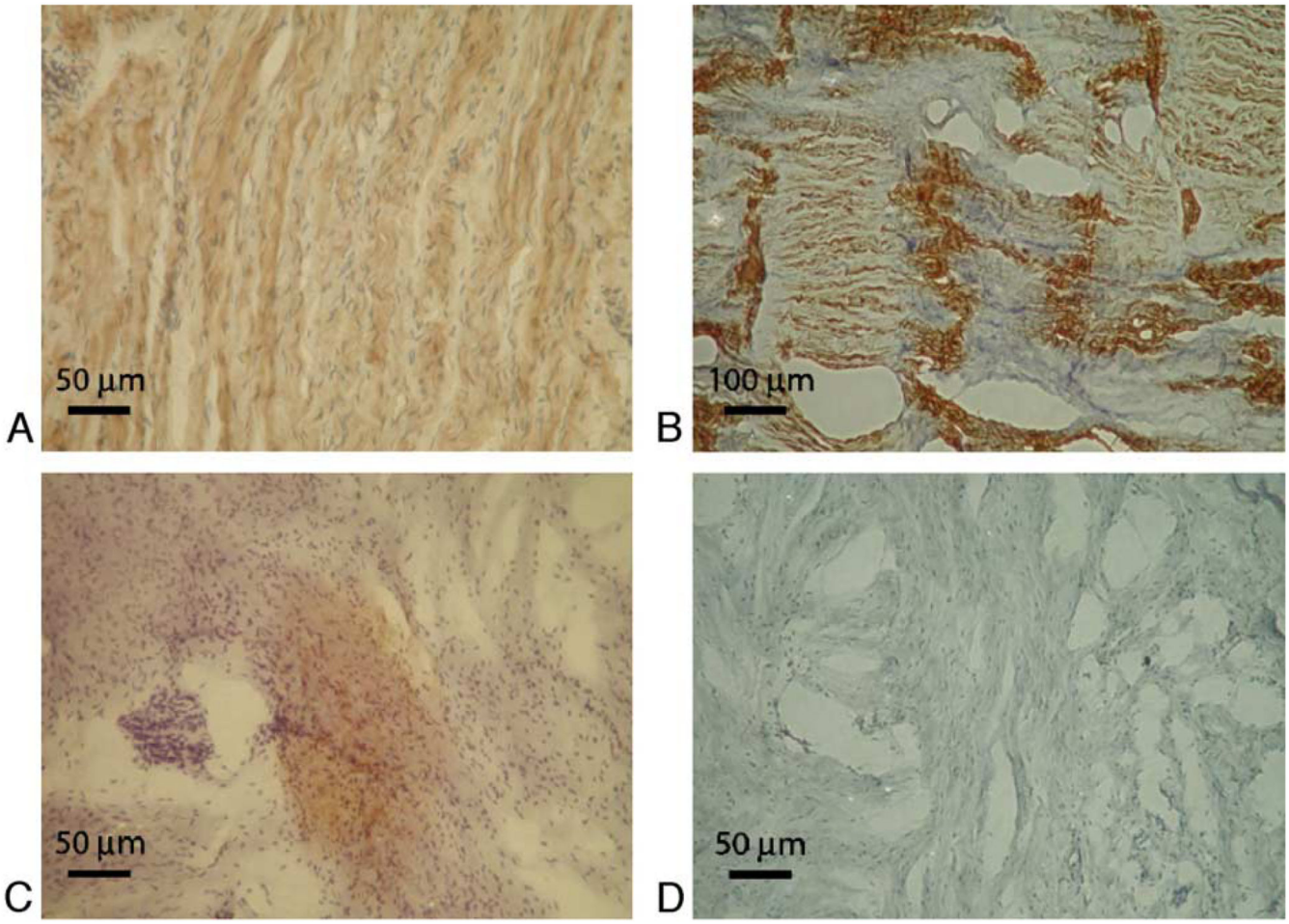

Fig. 5.

Collagen immunostaining. (A) Collagen I stain in the superior medial region of the anterior band. (B) Collagen I stain in the inferior medial region of the intermediate zone. (C) Collagen II stain in the superior lateral region of the intermediate zone. (D) Negative control for collagen I and II.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

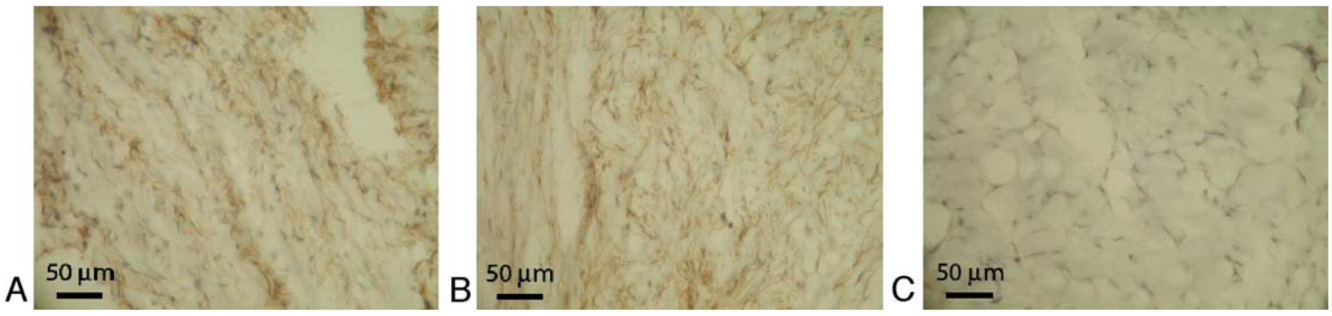

Fig. 6.

Elastin immunostaining in the (A) inferior lateral region of the intermediate zone and (B) superior central region of the posterior band. (C) Negative control.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

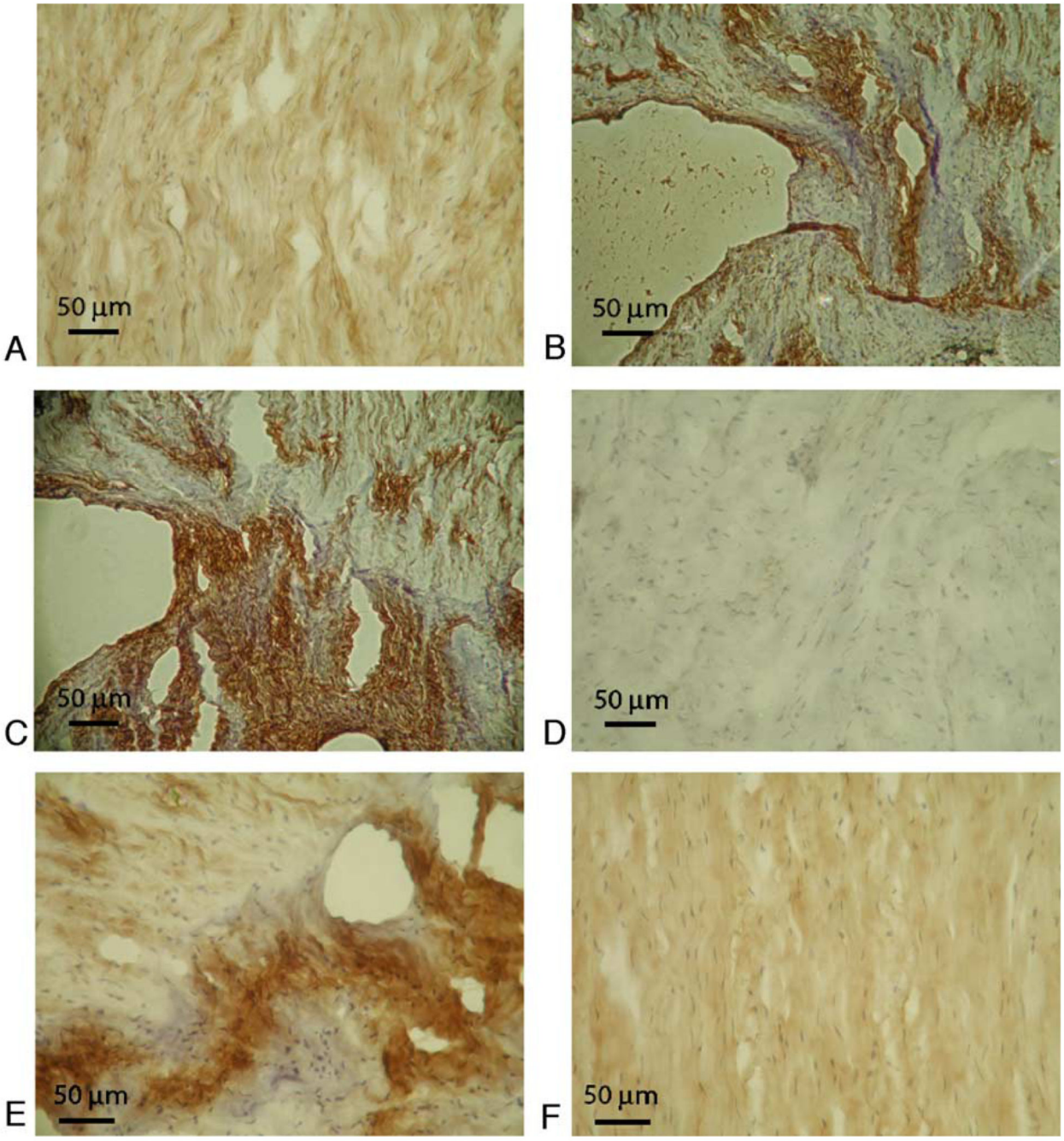

Fig. 7.

Chondroitin sulfate and keratan sulfate immunostaining. (A) Chondroitin-4-sulfate stain in the superior central region of the posterior band. (B) and (C) Serial sections in the superior medial region of the intermediate zone stained for chondroitin-4-sulfate and chondroitin-6-sulfate, respectively. (D) Negative control for chondroitin sulfates and keratan sulfate. (E) Keratan sulfate stain in the superior central region of the intermediate zone. (F) Keratan sulfate stain in the superior central region of the posterior band.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

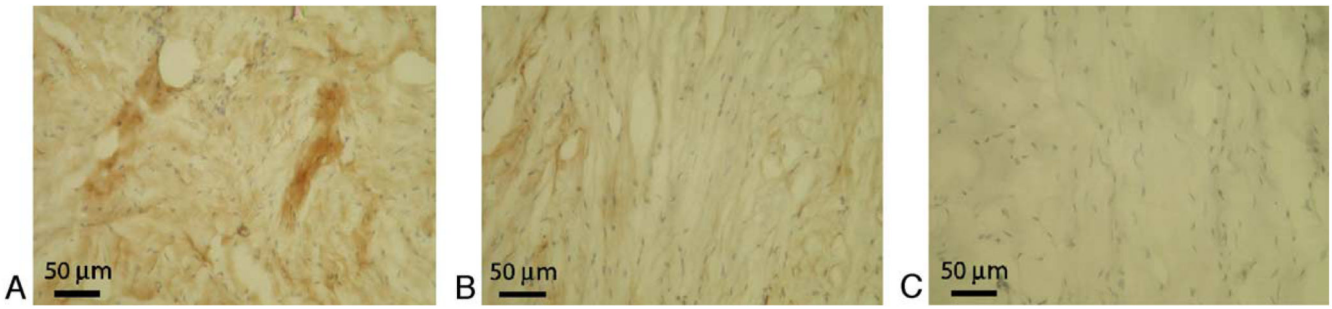

Fig. 8.

Dermatan sulfate immunostaining in the superior central regions of the (A) intermediate zone and (B) posterior band. (C) Negative control.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

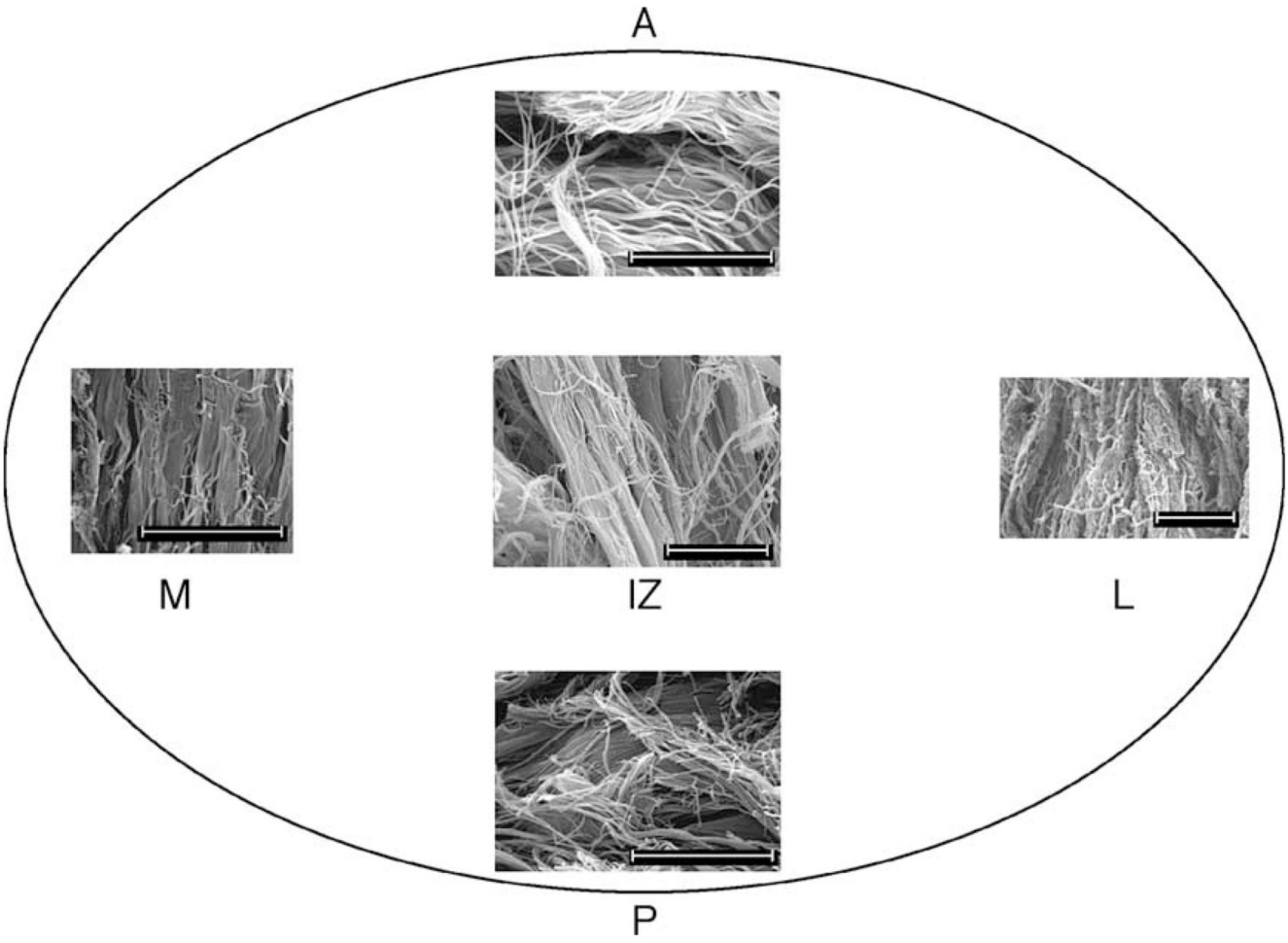

Fig. 9.

Scanning electron micrographs of representative regions of the TMJ disc. A: Anterior band, fibers running primarily in the mediolateral direction and also anteroposteriorly (3500×). IZ: Intermediate zone, anteroposterior orientation of collagen fibers (2500×). P: Posterior band, collagen fibers aligning in the mediolateral direction (3500×). M: Medial area, showing collagen fibers in a preferential anteroposterior direction (3500×). L: Lateral area, anteroposterior orientation of collagen fibers (100×). Scale bars denote 10 μm, except for the lateral region for which it denotes 200 μm.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Summary of significant GAG content regional variations

GAG	Anteroposterior	Mediolateral
Chondroitin-6-sulfate	IZ, A>P	M>C, L
Chondroitin-4-sulfate	IZ>A>P	M>L
Keratan sulfate	IZ>A, P	None
Dermatan sulfate proteoglycan	IZ>A, P	None
Hyaluronic acid	None	None

A—anterior band content, IZ—intermediate zone content, P—posterior band content, M—medial region content, C—central region content, L—lateral region content.

No significant superoinferior variations.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

ELISA protocol parameters

GAG/Proteoglycan	Coat concentration[a] (ng/mL)	1° Antibody dilution[b]	2° Antibody dilution	Sample dilutions			Standard range (μg/mL)
KS	1000	500	20,000	10	25	50	1–100
C4S	25	1200	20,000	100	250	500	1–100
C6S	25	5000	20,000	50	100	250	1–100
DSPG	1333	2000	16,000	1.5	3	9	25–500
HA	n/a	1000	30,000	2	35	100	5–200

KS—keratan sulfate, C4S—chondroitin-4-sulfate, C6S—chondroitin-6-sulfate, DSPG—dermatan sulfate proteoglycan, HA—hyaluronic acid.

[a] For KS, C4S, and C6S ELISAs, plates were coated with aggrecan. For DSPG ELISAs, plates were coated with decorin.

[b] Dilutions for inhibition ELISAs refer to the incubation period, after being combined with samples/standards.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3

Immunohistochemistry protocol parameters

ECM constituent	Tissue digestion[a]	1° Antibody dilution	2°Antibody dilution
KS	Chondxoitinase ABC, 0.2 U/mL, 2 h	500	300
C4S	Chondroitinase ABC, 0.2 U/mL, 2 h	100	300
C6S	Chondroitinase ABC, 0.2 U/mL, 2 h	100	300
DS	Chondroitinase AC, 0.1 U/mL, 3 h	5	200
HA	n/a	250	200
Collagen I	n/a	1500	300
Collagen II	n/a	1000	300
Elastin	Trypsin, 1 g/L, 10 min	100	300

KS—keratan sulfate, C4S—chondxoitin-4-sulfate, C6S—chondxoitin-6-sulfate, DS—dermatan sulfate, HA—hyaluronic acid.

[a]Chondroitinase solutions diluted in 0.1 M Tris-acetate.