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Biochemical and Histological Differences Between Costal and Articular Cartilages

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Abstract	<p>Biologically, costal cartilage is an understudied tissue type and much is yet to be learned regarding underlying mechanisms related to form and function, and how these relate to disease states, specifically chest wall deformity. Chest wall deformities have a component of inheritance, implying underlying genetic causes; however the complexity of inheritance suggests multiple genetic components. At our Centre investigations were performed on gene expression of key select genes from costal cartilage removed at surgery of patients with chest wall deformity to show high expression of decorin, a key player in collagen fiber formation and growth. Also, the degree of tissue differentiation was investigated that was different to that of articular cartilage as measured by gene ratio. Ultrastructural aspects of costal cartilage were determined by scanning and atomic force microscopy to show the presence of ‘nanostraws’ and preliminary data of nanostraw strength by measuring Young’s modulus of individual nanostraws. Protein deposition of collagen type II, decorin, and biglycan suggest orchestration of fiber formation in the interterritorial matrix. Although no specific biological markers related to chest wall deformity have currently been identified, work from our Centre has identified potential areas of interest.</p>	
Keywords (separated by “ - ”)	Costal cartilage - Chondrocytes - Collagen - Aggrecan - Decorin - Biglycan - SLRP - Glycosylation - Scanning electron microscopy - Atomic force microscopy - Gene expression	

Biochemical and Histological
Differences between Costal
and Articular Cartilages

Michael W. Stacey

Abbreviations

AFM	Atomic Force Microscope
cDNA	Complimentary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular matrix
FCD	Fixed Charge Density
GAG	Glycosaminoglycan
MMP	Matrix Metalloproteinase
PBS	Phosphate Buffered Saline
PC	Pectus Carinatum
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscope
SLRP	Small Leucine Rich Proteoglycan
SNP	Single Nucleotide Polymorphism
VNTR	Variable Number of Tandem Repeats

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Introduction

Costal cartilages, a type of hyaline cartilage, are bar like structures connecting the ribs to the sternum and allows for rib cage flexibility. Unlike articular cartilage of the joints, which is only a few mm thick, costal cartilage can approach approximately 1 cm in diameter. Like other cartilage types, the body of the tissue does not have nerve or blood supplies. Costal cartilage does have a surrounding perichondrium that is vascular and provides nutrients. Nutrients diffuse into the cartilage, but it is estimated that diffusion coefficients are in the order of 200 mm, a fraction of the diameter of costal cartilage. This results in a dilemma regarding costal cartilage structure and function. Centrally located cells are deprived of oxygen and cell metabolism creates an acidic environment. Indeed, chondrocyte gene expression is up regulated in these conditions, and needs to be accounted for in future experiments; however, cells still require some minimal nutrient and gas exchange to function. Our group examined costal cartilage immunohistochemically and by scanning electron microscopy to determine the structure and protein content of costal cartilage, and genetically by examination of gene expression of key cartilage related genes.

Cellular Distribution

Histologically, the proteins of cartilage are produced by chondrocytes that are sparsely distributed throughout the secreted extra cellular matrix (ECM), occupying only approximately 2 % of cartilage. Few studies have been undertaken on chondrocyte distribution within cartilage, yet cell density and arrangements are considered to be critical to function. Cellular clusters [1], pairs [2], and rows [3], have been reported. A more extensive study [4] in the superficial zone of articular cartilage identified complex patterns that appear to be location specific. For example, chondrocytes of the articular surface of ankle joints were as pairs, whereas strings of chondrocytes were found in half of the superficial chondrocytes of the femoral condyles of the knee joint, and circular clusters were found in the patella-femoral groove of the femur [4]. It has been suggested that chondrocyte groups form after cell division with incomplete separation within the cellular microenvironment allowing direct communication between cells [5]. In cross sections of costal cartilage removed from a patient with pectus carinatum we observed 98 % of cells were single or paired towards the periphery whereas 8 % appeared as clusters of 3–4+ cells in central regions, consistent with the notion that younger cells that have undergone one or no divisions are peripheral, with older cells having undergone more divisions being embedded more centrally. In a control sample, ~13 % of cells were in clusters of 3–4+ in central regions. Although the control sample showed cell clusters nearly twice that of a PC sample, verification of rib number and site was not possible, and thus in this instance site specific variation cannot be confirmed. No strings were observed in PC or control sample. A spatial relationship between collagen fiber alignment and cellular organization has been suggested [3], with chondrocytes running parallel to adjacent fibers. When we examined longitudinal sections of costal cartilage, we also note the presence of lacunae between the large fibrous structures [6]. The predominance of single and doublets in costal cartilage suggests cells undergo relatively few divisions. The absence of extensive strings and

clusters is likely due to the different biomechanical forces experienced by costal cartilage compared to articular cartilage of joints.

The forces experienced by costal cartilages are very different to those of load bearing cartilage and a comprehensive investigation of the cellular distribution along the length of individual costal cartilages may reveal important insights into apparent weaknesses observed in patients with chest wall deformities.

Costal Cartilage Structure

Collagen Type IIA1 (COL2A1) Presence in Costal Cartilage

Recent work [7] described a decrease in the biomechanical stability of costal cartilage in pectus excavatum patients and suggested a disorderly arrangement and distribution of collagen fibers. Other authors have suggested that atypical collagen fibers may be implicated in chest wall deformities [8, 9]. The arrangement of collagen fibers in costal cartilage has not been described in detail; however, highly ordered fiber formation was described in the surrounding perichondrium [10]. A lack of understanding of molecular and ultrastructural properties hampers understanding events leading to these disorders.

Collagen type II is a major constituent of hyaline cartilage, consisting of ~70 % of cartilage dry weight. It is a fibrous protein that is cross-linked to other fibers and adds structure and strength. Mutations in collagen genes have been described in many skeletal dysplasias, but no abnormalities have been described in patients with chest wall deformities. The presence of COL2A1 in costal cartilage is shown in Fig. 7.1. Staining appears to be intense surrounding cellular lacunae, with a more uniform staining pattern within the matrix. This staining is consistent with COL2A1 staining in hyaline cartilage. Negative controls showed no staining (data not shown).

Aggrecan Presence in Costal Cartilage

Aggrecan, a large proteoglycan present at 3–10 % cartilage dry weight, serves as an attachment for chondroitin and keratin sulphates,

Fig. 7.1 Immunohistochemistry using a COL2A1 specific human monoclonal antibody showing intense COL2A1 staining (arrows) around cellular lacunae (*), with a lighter, more uniform COL2A1 stain over the matrix (arrow heads) [44]

highly negatively charged proteins that are responsible for the fixed charge density of cartilage. This fixed charge density (FCD) is the source of all electrochemical events in cartilage [11] and is responsible for cation movement into the tissue along with water to produce osmotic swelling that gives cartilage its unique physical properties. Abnormalities in aggrecan can result in weakened cartilage, as exemplified in osteoarthritis where breakdown of aggrecan, reduced FCD, and associated reduction in osmotic pressure, result in weakened cartilage susceptible to wear and tear.

Hypoxia or low pH has been shown to act as a trigger for aggrecan production through induction of hypoxia inducible factor 1- α and *SOX9*, as well as inhibit *COL1A1* expression [12, 13]. Similarities with inter vertebral discs are noteworthy. Cells embedded within the centrally located nucleus pulposus experience hypoxia and express aggrecan under the regulation of the hypoxia induced P13K/AKT signaling pathways via modulation of *SOX9* [14]. It appears that as cells become centrally located they, due to lack of blood supply, experience hypoxia and lower pH. Consistent with this hypothesis, we observed aggrecan deposition by chondrocytes in centrally located regions of costal cartilage compared to those cells at the periphery (Fig. 7.2). The induced

negatively charged environment draws in water and cations, e.g., Na^+ that was confirmed by Electron Probe Micro Analysis in a section of costal cartilage (Fig. 7.3).

Although collagens and aggrecan are major protein constituents of cartilage, major differences between articular cartilage and costal cartilage in form and function are very apparent and thus major differences in protein content and deposition are expected. The presence and driving force of aggrecan and associated negatively charged proteins are common to both; however the arrangement of collagen fibers in response to their unique sites primarily reflect function. This draws the question as to how fibers are arranged in costal cartilage, and what proteins may play a role in the process of fibrillogenesis.

Small Leucine Rich Proteoglycans (SLRPs)

Small leucine rich proteoglycans (SLRPs) are extra cellular matrix molecules that bind strongly to collagen and other matrix molecules. They are associated with collagen fibril formation and therefore important in the proper formation of ECMs. The co-operation, sequential, timely, orchestrated action of SLRPs that shape architecture and mechanical properties of the collagen matrix and overall importance in disease is reviewed by Ameye and Young [15]. Indeed, SLRP knockout mice exhibit disorganized collagen fibers and loss of connective tissue function [16, 17]. Fragmentation of SLRPs is associated with degeneration of matrix in meniscus, knee and hip cartilage [18]. The name arises from their relative small size of approximately 40 kDa compared to ~200 kDa for the large aggregating proteins aggrecan and versican, the presence of numerous adjacent leucine rich repeats and one or very few glycosaminoglycan (GAG) side chains. SLRPs are generally expressed in a very tissue specific manner. Mechanistically, it is accepted that horseshoe shaped SLRPs interact with individual collagen fibrils by their concave surfaces, and the space inside the horseshoe accommodates a single tri-

AU6

AU2

Fig. 7.2 Localization of aggrecan by immunohistochemistry in transverse cross-sections of costal cartilage. (a) Distribution of aggrecan in whole control section. (b) Distribution of aggrecan in whole PC3 section. (c–e) Distribution of aggrecan in control at 10× magnification from (c) periphery, (d) midzone, and (e) interior regions.

Scale bars, 100 μm. (f–h) Distribution of aggrecan in control at 100× magnification from (f) periphery, (g) midzone, and (h) interior regions. Scale bars, 10 μm. Notice the localization of aggrecan becomes more intense around each lacuna in the interior compared to the periphery

ple helix of collagen [15]. Mutations in SLRPs may be important as predisposing genetic factors for diseases of the ECM.

Reports of decreased biomechanical stability of costal cartilage in pectus excavatum and suggested disorderly arrangement, distribution, and atypical collagen fibers in chest wall deformities [7–9], led us to examine the size and distribution of two SLRPs expressed in cartilage, decorin and biglycan [19]. Both regulate ECM growth and fibrillogenesis. Interestingly, both sequester TGFβ, controlling availability of this growth factor and thus growth of cartilage. The important roles of decorin and biglycan in fibrillogenesis and sequestration of TGFβ makes them interesting molecules to investigate in disorders related to abnormal cartilage growth and strength. There may be a mechanistic role for these two SLRPs in

chest wall deformity. Decorin and biglycan are prominent class I members of the SLRP family and are homologous (57% identity at the amino acid level) but with divergent patterns of expression. Despite their similarities, biglycan and decorin have distinct functions which may partially result from differences in GAG chains; biglycan has two chondroitin sulphate chains and decorin has one, respectively (Fig. 7.4a, b).

Biglycan deficiency has been shown to cause spontaneous aortic dissection and rupture in mice [20], a characteristic of Marfan syndrome, a syndrome known to exhibit chest wall deformities. Decorin function is consistent with functions related to fibrillogenesis [17, 21]. Null mutations lead to abnormal collagen architecture in mice suggesting a mechanistic role for these proteins in skeletal disorders.

Fig. 7.3 Electron probe microanalysis of 100 equally spaced points over a transverse section of costal cartilage. Sodium is present only in the central 48 points (boxed), and not at the peripheral points, which contain Sulfur only (circled) showing that positively charged ions were drawn to central regions to achieve electroneutrality [6]

Decorin and biglycan have different isoforms, the pro and mature forms that show differential expression [22–24]. Proforms have a 14 amino acid N-terminal pro-peptide that is cleaved in the mature forms. The abundance of pro-forms of both SLRPs is tissue and age dependent, with the mature form being expressed more highly in juvenile tissue, and the pro form in adult [25, 26]. Structurally, costal cartilage has been shown to consist of straw-like structures running parallel to the length of the tissue. Gene expression in this tissue shows high levels of decorin compared to biglycan [6]. The complex arrangement of fibers observed in costal cartilage and the role of decorin and biglycan in these structures has not been explored. We investigated the presence and distribution of the different isoforms of decorin and biglycan. This was achieved by immunohistochemistry of costal cartilage from teenage patients with pectus carinatum and an age-matched control using antibodies to the different isoforms of decorin and biglycan [19, 22]. Our results (Fig. 7.5) show the presence of mature form of decorin and pro-and mature forms of biglycan in the interterritorial matrix in patient and

control samples. Prodecorin was localized only to the cells [19].

No apparent differences were observed between an age-matched control and patient samples; however, proforms of decorin and biglycan are maintained evolutionarily, suggesting they play an important, undetermined, functional role.

Overall immunohistochemistry shows intense perilacunae staining for collagen type II, mature biglycan, and mature decorin. This is indicative of orchestration of the ECM production occurring soon after proteins are secreted from the cell. Biglycan likely initiates collagen fibril organization that is further assembled by decorin to form the large nanostraw-like structures characteristic of costal cartilage. Many other proteins will play a crucial role in the final network [19, 27]. Decorin and biglycan sequester TGF β , controlling availability of this growth factor to chondrocytes. This needs to be proven in the environment of costal cartilage as it assumes the presence of this growth factor within the matrix, and thus a means of transport to arrive there. The components of decorin and biglycan, (leucine rich repeats, absence or presence of propeptide, and variable O-and

Fig. 7.4 A schematic showing salient features of biglycan and decorin

Fig. 7.5 Detection of biglycan (LF-112, Fisher et al. 1996) (Bar 50 μm) and decorin (LF-136, Fisher et al. 1996) (Bar 100 μm). Both show dark staining around

lacunae, however decorin appears to be slightly more intense and more widely distributed

N-linked glycosylated side chains), allow for multiple interactions with collagen fibrils and may be an important factor in cartilage stability in relation to formation of chest wall deformities. Careful analysis of the composition of GAG side-chains may be warranted as the sugar content of such chains has been suggested to play a role in binding of collagen fibers and pathology [28].

Glycosylation

Increasing attention is being given to the glycosylated side chains of proteins, particularly with relevance to disease [29]. Differences in side chains of decorin and biglycan have been described from different cartilage sites, including non-glycosylated decorin and biglycan in nucleus pulposus of intervertebral discs [30]. Decorin has a single O- and three N-linked glycosylation sites. Biglycan has two O-linked and two N-linked sites. O-linked sites are typically covalently bound by chondroitin/dermatan sulphate (Fig. 7.4). Dermatan sulphate is linked to these molecules in skin where defects of glycosylation have been described in Ehlers Danlos syndrome underlying collagen fibrillogenesis [28]. In cartilage chondroitin sulphate is covalently bound in the O-position. Glycanated side chains show length variation, with shorted chains being associated with tighter collagen fiber configuration [31]. More recent work [32] has shown that decorin may bind to one collagen fibril by its core protein and to another by its side chain. Additionally, they demonstrate that closely related side chain molecules (chondroitin-4-sulphate and chondroitin-6-sulphate) have very different effects affecting fusion and layout of collagen fibers. This exemplifies the importance of recognizing subtle variations in these molecules and their biological consequences in addition to enzyme systems that are responsible for synthesis and assembly of these molecules. Sulphate anion transporter abnormalities have been described in chondrodysplasias [29]. Interestingly, a recent report [33] describes a mutation in the GAL3ST4 gene in a single Chinese family showing dominant inheritance of

pectus excavatum. GAL3ST4 is a member of the sulfotransferase family that catalyzes the C-3 sulfation of galactoses in O-linked glycoproteins. Sulfation of proteoglycans is crucial for normal development of bone and cartilage [34], and defects in genes encoding catalytic machinery responsible for sulphate biosynthesis have been reported [35, 36]. The inheritance of chest wall deformity is extremely complex [19, 37, 38], however the importance of the role of enzymes responsible for glycosylation cannot be overlooked and this report [33] may be corroborated with other genes on these pathways.

Scanning Electron Microscopy

Suggestions that atypical collagen fibers may be implicated in chest wall deformities led us to investigate ultrastructural aspects of costal cartilage. Scanning electron microscopy (SEM) was undertaken on a transverse section of costal cartilage to investigate distribution of collagen fibers in this tissue. Figures 7.6a, b are representative SEM images of a transverse cross-section of costal cartilage. Figure 7.6a shows a fracture in the cartilage exposing collagen fibers of approximately 600 nm diameter. Fibers come together to form an extremely large complex of many μm (arrowed) that run parallel to the length of the cartilage. Figure 7.6b is a higher magnification of the boxed area and shows that each fiber forms a nanostraw of approximately 650 nm external diameter and 250 nm internal lumen diameter. Images of longitudinal sections show a well-defined organization with bundles of collagen fibers of approximately 20 μm diameter and cellular lacunae, arrowed in Fig. 7.6c.

We measured the diameters from 150 clearly defined fibers from SEM images and found that most (51.1%) were in the range from 1 to 100 μm . The smallest ($<0.1 \mu\text{m}$) would most likely represent collagen fibrils, the midsize ($\sim 1 \mu\text{m}$) would represent the microtubes and the largest ($\sim 100 \mu\text{m}$) would be large fascicle-like structures [6].

This work shows unique ultra-structural properties of costal cartilage. The presence of

Fig. 7.6 SEM images of normal costal cartilage. (a) Transverse section (×2500) showing large numbers of dense fibrils running longitudinally (*arrowed*). (b) Magnification (×10,000) of the boxed area in a and shows the presence of collagen nanostraws (*arrowed*). Each

straw is approximately 650 nm in diameter, with a lumen diameter of approximately 250 nm. (c) Longitudinal section (×500) showing large bundles of collagen fibers, formed from multiple collagen nanostraws, of approximately 20 μm diameter (*white arrow*)

straw-like structures shows that a large degree of complex extracellular matrix formation occurs. Form and function are inextricably linked in biology and the role of these structures remains to be verified. Bundles of fluid filled straws would certainly add strength while allowing flexibility during movement. Indeed, movement may be a driving force for fluid transport within costal cartilage, allowing some degree of nutrient and gas exchange for internally located cells. Assuming that the strength of costal cartilage is related to the sum of individual nanostraws, or conversely that weakness may be reflected in a more deformable nanostraw, we set out to determine mechanical properties of individual nanostraws. Young's modulus is a means to measure the elastic properties of materials that are stretched and compressed and can be described as

Stress (N/m²) expressed as force (F)/area (m²)
Strain (m/m) expressed as elongation or compression of object (dL)/length of object (L).

Young's modulus = stress/strain = (F/A)/(ΔL/L)

Stress and strain are resisted by collagen fibers and changes in the properties of collagen fibers would influence Young's modulus. Many pathological processes change tissue elasticity and is the basis of palpitation as a diagnostic tool. Some interesting values for Young's modulus are given in Table 7.1, where low values are derived from compliant materials, and high values from resilient material.

Cartilage values in Table 7.1 are for articular cartilage, however, reported values depend very much on biological sample preparation and measurement technique [39]. Our rationale for atomic

Table 7.1 Example of a range of Young's moduli from compliant rubber to hardened steel [6]

Material	Approximate Young's modulus (10 ⁹ N/m ² ; GPa)
Bone	9
Cartilage	2.4
Tendon	5.5
Rubber	0.01–0.1
Pine wood	9
Stainless steel	180

force microscopy was that individual nanostraws would have characteristic Young's modulus of elasticity, and that these may be reduced in samples from patients with chest wall deformities due to abnormalities in the assembly of nanostraws. Costal cartilage from patients with chest wall deformities have often been described as weak, particularly in those who do not do well in surgery.

Atomic Force Microscopy

The atomic force microscope (AFM) is a very high resolution scanning probe microscope that has found applications from the biological to the material sciences and has several advantages over transmission and scanning electron microscopy, including the absence of electron-induced specimen damage, ambient operation, preservation of biological morphology, and the ability to be utilized on live or fixed tissues. Analysis of biological samples frequently necessitates their fixation and protein cross-linking by chemical fixation, although fixation itself can cause tissue distortion. The AFM probes the surface topography of a sample to a very high resolution irrespective of whether the tissue is live or fixed. Probing of live tissues opens the possibility of investigating biomechanical measurements, for example, Young's modulus of elasticity [40–42]. Because AFM probing can be undertaken when the sample is submerged, it is possible to maintain live samples under physiological conditions.

It was proposed that the straw-like structures observed in costal cartilage act as a means of nutrient and gas transport; additionally they provide biomechanical support [6]. This is analogous to the pressure induced fluid flow in the canaliculi-lacunae network described in bone [43]. Here stress induced microcirculation in canaliculi of approximately 200 μm in diameter was investigated to show that flow can nourish 4–5 layers of concentric osteocytes and also suggest that stress induced flow may be important in bone remodeling where lack of flow may have pathological consequences, e.g., osteoporosis. Further characterization of collagen nanostraws is war-

ranted if such a model is to be applied to fluid flow in costal cartilage.

In order to further characterize collagen nanostraws, brief homogenization and enzymatic digestion of cartilage with trypsin and hyaluronidase was used to isolate individual samples [44]. Individual nanostraws were examined for D-zone spacing and Young's modulus of elasticity. D-Zone bands are characteristic of collagen fibers, reflect the underlying regular arrangement of fibrils, and are estimated to be approximately 67 nm in hydrated and 64 nm in dehydrated samples [45]. The D-Zone patterns were measured from an SEM image compared to a digested and homogenized AFM image in air (Figs. 7.7a, b, respectively) and found mean D-Zone values of 63 nm and 65 nm from 10 zones each. These results are consistent with shorter D-Zones in dehydrated collagen forms suggesting that the underlying arrangement of fibers in costal cartilage derived from a patient with pectus carinatum is comparable to normal values under these conditions.

To determine Young's modulus of elasticity, individual isolated nanostraws were attached onto poly-L-lysine cover glass. Force measurements were performed using frequency modulation force spectroscopy [46], and the resulting force data was modeled using the Derjagin, Muller, Toropov (DMT) model [47–49] via an in-house data analysis program written in MATLAB® (version 2009, Mathworks). Figure 7.8 shows typical force measurement on a nanostraw for digested, homogenized and fixed specimen in air. Utilizing the DMT model the modulus of elasticity from six separate measurements is found to be 2.06 ± 0.35 GPa.

Collagen nanostraws are structures significantly larger than individual collagen fibers and may be cross-linked by many structural proteins. Force measurements published in the literature, conducted for dehydrated collagen fibrils obtained from the common sea cucumber and analyzed at ambient conditions, resulted in values ranging between 1 and 11.5 GPa [50–52]. These values are high compared to reported hydrated, unfixed samples, where values of 2–5 MPa are reported [53]. These values strongly

Fig. 7.7 SEM (a) and AFM (b) of characteristic D-zones of collagen fibers. A single D-zone is blocked and arrowed in a. The insert in b shows variation in D-zones over a single fiber [44]

depend upon ionic concentration, hydrogen bonding, and hydration forces, all of which can influence interactions of tropocollagen molecules and, therefore, the elastic modulus. High values observed in Fig. 7.8 from costal cartilage (23 MPa) are derived from fixed samples, and due to cross-linking of proteins, create a more ridged structure and thus a higher Young's modulus.

Overall, these results show the unusual tubular network of costal cartilage that is hypothesized to act as a means of fluid and gas transport. To study nano-fluidic transport, such structures necessitate the accurate measurements of their dimensions. Interestingly, previous reports suggest that in rabbit tibia these structures correspond to the known biomechanical properties of the tissue, and would act as a dampening system during compression by resisting lateral fluid flow in the tissue and directing it against the compressive force [54]. Our study demonstrates that the protocols adopted for these measurements have significant influence on size measurement. Clearly, costal cartilage has large fiber dimensions with complex structures that are formed through finely tuned fibrillogenesis that ultimately reflect the biology of this understudied tissue type. The complex inheritance of chest wall deformities suggests that these processes are under the control of many genes.

Analysis of Candidate Genes

Aggrecan is an integral part of cartilage and mutations in the *ACAN* gene are associated with skeletal dysplasias [55, 56]. Patients with pectus excavatum commonly exhibit scoliosis, and *ACAN* has been investigated as a candidate gene in familial idiopathic scoliosis [57, 58]. The CS1 domain of the *ACAN* gene exhibits length polymorphisms due to a variable number of tandem repeats (VNTR), 19 amino acids in length. Each repeat acts as an attachment site for chondroitin sulphate [59]. The number of *ACAN* VNTRs determines the number of GAG side-chains. The presence on aggrecan of a large number of highly charged chondroitin sulphate chains generates an osmotic swelling pressure and is important in maintaining structural integrity of the tissue. Smaller repeat sequences may result in mechanical shearing and tearing [60], and are associated with rheumatoid arthritis and spinal disc degeneration [56, 61]. It was hypothesized that abnormalities of costal cartilage in patients with pectus excavatum may be due to variation in number of repeat sequences outside of the normal reported range of 26–28 [59, 62] that would result in a concomitant change in chondroitin sulphate anchorage sites and compromised structural characteristics.

For this investigations were performed on the size and frequency distribution of *ACAN* VNTRs

Fig. 7.8 (a) Representative topography of costal cartilage digested and homogenized. Contrast covers height variation of 390 nm. *Insets* in the figures show height distribution of nanostraws at various locations. (b) Force versus indentation depth data on a nanostraw for homogenized

digested fixed sample in PBS buffer. Analysis based on the DMT model gives the modulus of elasticity of $E = 23 \pm 3$ MPa in PBS buffer. Experimental data is shown by symbols, while the curve-fit of data to the DMT model is shown by *solid lines* [44]

in patients with pectus excavatum and correlated overall allele sizes (genotype) to Haller index (Fig. 7.9) [63]. This was achieved by isolating DNA from venous blood of patients or by isolation from chondrocytes derived from patient costal cartilage and amplifying VNTR regions by polymerase chain reaction (PCR) [59, 62].

Genotyping identified 15 alleles ranging from 19 to 34 repeats, with alleles 25–28 accounting for 94 % and 84.7 % respectively in patients and controls. Allele distribution differed between patient and control groups ($\chi^2=48.58$, $p<.009$) such that patients had 0.43 fold fewer 25 repeats ($\chi^2=7.41$, $p<.025$) and 1.5 fold more 27 repeat alleles ($\chi^2=145.32$, $p<.001$) compared to controls. Overall, however, we observe an allele frequency of 0.120, 0.866, and 0.014 in patients for <26, 26–28, and >28 alleles respectively, consistent with the normal observed range [59, 62]. There is no apparent bias of allele genotype with increased Haller index, therefore a specific combination of VNTRs does not predispose to increased severity in pectus excavatum.

Patients showed phenotypic variation, and subgroups were identified where a genetic component may be influential. Females (16 % of patients), showed a significant increase in severity compared to males ($t(250)=2.36$, $p<.019$; Mean+SD: 5.7+2.1 vs. 4.8+2.2), and tended to have a decreased number of VNTRs, consistent

with a hypothesis of reduced attachment sites for chondroitin sulphate and weakened cartilage.

Marfan phenotype patients (10.4 % of patients), exhibited phenotypic findings consistent with Marfan appearance such as long limbs, arachnodactyly and high-arched palate without absolute diagnostic criteria for Marfan syndrome [64]. There was no apparent correlation in the number of VNTRs compared to the non-Marfan patients, suggesting that VNTRs do not have a differential role in this subgroup.

Repeat surgeries are the smallest subgroup (3.2 % of patients) and showed no correlation between Haller index and surgical outcome, suggesting initial presentation is not an indicator of outcome.

Furthermore variation in a functional VNTR were investigated and identified, a useful marker for first-pass analysis. Investigation of SNPs will allow a more refined description in the inheritance of this, and other candidate genes, in the role of inherited chest wall deformities.

Analysis of Gene Expression in Costal Cartilage

Cartilage formation is a complex process with many interacting components. To determine gene expression in costal cartilage investigations were performed on twelve candidate genes based upon

Fig. 7.9 Correlation of aggrecan genotype (VNTR repeats/allele) with Haller index in male (squares) and female (diamonds) patients [63]

structural and functional importance in cartilage formation. Table 7.2 lists each gene and chromosomal location. Chest wall deformities show a sex bias, being more prevalent in males compared to females (4:1) [37, 38], suggesting that genes located on chromosome X may be of importance. Males have a single X chromosome (XY) and therefore defects in genes on this chromosome cannot be compensated for by genes at a second allele as in females (XX). Four genes were identified on chromosome X with relevance to cartilage formation (Table 7.2).

Aggrecan: A large aggregating proteoglycan that serves to anchor highly negatively charged keratin and chondroitin sulphate molecules ultimately responsible for generating osmotic pressure within cartilage.

Biglycan: *BGN*, a SLRP on the X-chromosome that encodes for the protein involved in assembly of collagen fibrils within the extracellular matrix of cartilage. It is closely related to decorin, possibly through gene duplication, and carries two glycosaminoglycan side chains. It strongly binds the growth factor TGF- β , controlling bioavailability.

Tissue inhibitor of metalloproteinase-1: *TIMP1* is located on the X chromosome and plays a role in the maintenance and turnover of the extracellular matrix within cartilage. It functions as an inhibitor of matrix metalloproteinases (MMPs), specifically MMP-8 and MMP-13, which are both collagenases.

Voltage-gated calcium channel- $\alpha 1F$: *CACNA1F* is a gene that encodes for a voltage-gated calcium channel and is found on the X chromosome. It functions to control the amount of calcium that enters the cell upon membrane polarization and may be linked to bioelectric components of cartilage.

Nyctalopin: The *NYX* gene was investigated because of its location on the X chromosome and its function as a SLRP. It is associated more with eye function, and defects in the gene result in a number of eye related anomalies including night blindness

Collagen α -1 chain: *COL1A1* encodes for Type I α collagen fiber found in most connective tissues. Although not expressed highly in articular cartilage, it acts as a marker of cartilage differentiation.

Collagen type II α -1: *COL2A1* encodes for collagen Type II- α fibers found in cartilage where mutations in this gene have been associated with chondrodysplasias. It is highly expressed in articular cartilage and is essential for cartilage to resist compressive forces.

Decorin: *DCN* is a SLRP that plays a role in matrix assembly. It has an important role in binding collagen fibrils and strongly influences fiber size and shape. It has a single glycosaminoglycan side chain. It binds to COL1A1, COL2A1 and the growth factor TGF- β , controlling bioavailability.

Fibrillin 1: *FBN1* encodes a large matrix proteoglycan that serves as a structural component in

Table 7.2 Candidate genes investigated in this study [6]

Gene	Name	Chromosome location
<i>ACTB</i>	β -Actin	7p22
<i>ACAN</i>	Aggrecan	15q26.1
<i>BGN</i>	Biglycan	Xq28
<i>CACNA1F</i>	Voltage-gated calcium channel- $\alpha 1F$	Xp11.23
<i>COL1A1</i>	Collagen α -1 chain	17q21.33
<i>COL2A1</i>	Collagen type II α -1	12q13.11
<i>DCN</i>	Decorin	12q21.33
<i>FBN1</i>	Fibrillin 1	15q21.1
<i>NYX</i>	Nyctalopin	Xp11.4
<i>SOX9</i>	SRY (Sex determining region Y)-box 9	17q24.3
<i>TGFβ1</i>	Transforming Growth Factor- β 1	19q13.2
<i>TIMP1</i>	Tissue inhibitor of metalloproteinase 1	Xp11.23

force bearing microfibrils and binds to TGF-beta. Mutations in this gene are associated with Marfan syndrome where chest wall defects are common. One of these mutations creates an N-glycosylation site that disrupts multimeric assembly [28].

Sex determining region SRY box-9: SOX9 is a homeobox class of DNA binding proteins. It is a potent activator of *COL2A1* and may also regulate the expression of other genes involved in cartilage formation by acting as a transcription factor for these genes.

Transforming Growth Factor-β1: TGFβ1 is a multifunctional protein that controls proliferation and differentiation in many cell types. It regulates many other growth factors, and stimulates chondrocyte cell growth through the MAPK3 signaling pathway.

β-Actin: ACTB, was used throughout as a reference housekeeping gene and from which relative levels of gene expression were calculated.

For this, costal cartilages were immediately placed into a solution of RNAlater after surgery to preserve the integrity of expressed genes. RNA was extracted as described previously [6]. RNA was reverse transcribed to produce cDNA and amplified by RT-PCR on a BioRad CFX96 real time system (Fig. 7.10). Gene expression was measured by incorporation of SYBR green into

amplified products. All primers were designed specifically for gene amplification (Qiagen CA, USA). Relative fold differences in gene expression were calculated as $2^{-(CtGOI - CtHKG)}$, where CtGOI is the Ct value of the gene of interest compared to the CtHKG, which is the Ct value for the house keeping gene [65].

Costal cartilage from individuals with chest wall deformities is described as abnormally grown and weak. Typically, surgical repair takes place during teenage years to early 20s. Phenotypically, there is considerable variation of the clinical condition of PC, reflecting the complex nature and inheritance observed in these families. Variation in gene expression between samples is, therefore, expected; however, it is unknown whether the expression of matrix genes will be affected by surgical procedures. We compared gene expression of 4 patients with pectus carinatum to an age-matched-control. *COL2A1*, *DCN*, *ACAN*, and *TIMP1* are all highly expressed compared to *ACTB*, however, when normalized to control (=100%) significant reductions in expression are observed with sample variation (Table 7.3).

Compared to control, PC1 showed significant reduction in expression of *DCN* ($p < 0.001$) and *TIMP1* ($p < 0.001$). PC3 showed significantly lower expression of *COL2A1* ($p < 0.001$) and like PC4, both showed decreased expression of *ACAN* ($p < 0.03$ and $p < 0.024$, respectively). PC4 also

Fig. 7.10 Gene expression curves showing left to right, expression of *DCN*, *TIMP-1* (overlapping *actin*) *ACTB*, *BGN*, and *TGFβ*. High expression is displayed as a curve farthest to the left and lower expression moving to the right

showed significantly higher expression of *TIMP1* ($p<0.001$) and decreased expression of *BGN* ($p<0.04$). PC2 showed significant reduction in expression of *COL2A1* ($p<0.01$), *DCN* ($p<0.0002$), *TIMP1* ($p<0.001$), *BGN* ($p<0.03$) and *FBN1* ($p<0.01$). This sample, like all PC samples, was immediately processed from the operating room, although results suggest possible degradation of this sample.

Many patients with chest wall deformities are considered Marfanoid-like [64] without fulfilling all criteria for diagnosis of Marfan syndrome, including mutations of the fibrillin-1 gene. The expression of this gene was not significantly different between control and patients, with the exception of PC2 ($p<0.01$). Expression of the X-linked genes *NYX* and *CACNA1F* was not detected in any samples. Overall, deregulation of *TIMP1* expression was evident in 3/4 PC samples, and expression of *DCN* was significantly lower in 2/4, suggestive of roles for fibrillogenesis and matrix turnover.

Table 7.3 Percent fold difference in gene expression of four patients with pectus carinatum compared to β -actin and normalized to an age-matched control

	PC1	PC2	PC3	PC4
<i>COL2A1</i>	100	*63	*35	89
<i>ACAN</i>	122	93	*42	*205
<i>DCN</i>	*25	*25	92	117
<i>TIMP1</i>	*21	*29	107	*174
<i>ACTB</i>	100	100	100	100
<i>BGN</i>	43	*24	60	*22
<i>COL1A1</i>	125	100	92	100
<i>FBN1</i>	NA	*39	107	65
<i>SOX9</i>	91	61	46	191
<i>TGF-β1</i>	83	72	50	22

Significant differences in expression between control and patients are marked with * [6]

The differentiation status of cartilage can be equated to the ratio of *COL2A1*, present in differentiated cartilage, to *COL1A1*, present at higher levels in more undifferentiated cartilage. We compared ratios of gene expression from our samples to published data.

Ratios of the differentiation markers *COL2A1:ACAN* and *COL2A1:COL1A1* are low in PC patients and control (Table 7.4) compared to rabbit articular cartilage (1090 and 1790, respectively) but both are highly comparable to the nucleus pulposus region of lumbar discs (23 and 930 respectively), [66]. The ratios of *ACAN:COL1A1* fall between those reported for fully differentiated rat chondrosarcoma cells (78.4) and dedifferentiated chondrocytes cultured from costal cartilage (4.6) [67]. A high expression ratio of *COL2A1:COL1A1* (294.6) in human articular cartilage has been reported [65], but here results are referenced to *GAPDH* rather than *ACTB*. Overall, these results suggest costal cartilage is at an intermediate stage of differentiation and likely represents the different functional requirements of this tissue compared to articular cartilage. The differentiation similarities between lumbar discs and costal cartilage are of interest. The high incidence of scoliosis in patients with chest wall deformities indicates that defects of cartilage of a specific differentiation status may be very important. Small differences exist however between patients and between patients and control (Table 7.4), suggesting that gene ratios measured here are not major contributors to chest wall abnormalities in these samples. Interestingly, *DCN* is expressed at high levels compared to *BGN*. As well as binding growth factors, both SLRPs have a role in fibrillogenesis and were hypothesized to play a role in the etiology of chest wall deformities. The high *DCN/BGN* ratio strongly suggests the importance of decorin

Table 7.4 Gene expression ratios in costal cartilage from pectus carinatum and age-matched control

	<i>COL2A1/ACAN</i>	<i>COL2A1/COL1A1</i>	<i>ACAN/COL1A1</i>	<i>DCN/BGN</i>
PC1	35	878	25	8
PC2	29	701	24	13
PC3	36	427	12	19
PC4	19	990	53	69
Control	43	1117	26	13

expression in costal cartilage morphology. Decorin is present at high levels during tendon (fibrocartilage) development and persists until thick fibers are formed [17], thus parallels with costal cartilage (hyaline cartilage) are apparent.

Conclusions

Biological properties of human costal cartilage are a much understudied field. In this chapter preliminary data that investigates these properties were described. Sample characterization is of utmost importance and future studies should attempt to utilize samples from different but identified ribs, and the site of control samples should be verified for comparative purposes. Acquisitions of healthy age match controls are not easy because the age of patients tend to be teens to twenties. It has been suggested that rib abnormalities may be secondary to events of the thorax, with costal cartilage responding to micro-environmental factors, changing their biological characteristics as a result. The 'chicken and egg' paradox needs to be resolved, and identification of biological causes identified. This is particularly relevant to patients who do not do well in surgery, where a biological basis may underlie their prognosis and outcomes.

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