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Patterns of Gene Expression from Human Costal Cartilage in Relation to the Chest Wall Deformity Pectus Carinatum

Janna E. Grubbs
Old Dominion University

PATTERNS OF GENE EXPRESSION FROM HUMAN COSTAL CARTILAGE IN RELATION TO THE CHEST WALL DEFORMITY, PECTUS CARINATUM

by

Janna E. Grubbs
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Approved by:

Chris Osgood (Director)

Michael Stacey (Member)

Emilia Oleszak (Member)

ABSTRACT

PATTERNS OF GENE EXPRESSION FROM HUMAN COSTAL CARTILAGE IN RELATION TO THE CHEST WALL DEFORMITY, PECTUS CARINATUM

Janna E. Grubbs
Old Dominion University, 2013
Director: Dr. Christopher Osgood

Cartilage deformities within the human chest wall, specifically pectus excavatum (PE) and pectus carinatum (PC) are common (1/400-1/1000) and yet, despite their clinical significance, are some of the least studied disorders pertaining to cartilage [1]. The costal cartilage connecting "false ribs" 8-10 to the sternum is often abnormally grown and can lead to formation of a severely sunken "funnel" chest (PE) or push outwards to form a "pigeon" chest (PC). Both conditions can have impact on the diaphragm, heart, lungs, and psychological function. An established ratio of PE and PC in males to females is 4:1, indicating a sex-linked male prevalence to these disorders.

In this study, costal cartilage samples from patients with PC were examined in comparison to samples from control cartilage, i.e., from those who did not have other known chondral dysplasias. We hypothesized that significant differences in expression of key cartilaginous related genes between the PC patients and an age-matched control would be observed. The genes of interest included *COL1α1, COL2α1, SOX9, ACAN,* compared to a housekeeping gene, Actin *(ACTβ).* We also analyzed expression of the small leucine-rich repeat proteoglycans (SLRPs) *BGN* and *DCN,* which bind to collagen fibers located in the extracellular matrix. The potent transcription factor of chondrogenesis studied was *TGFβ1,* as well as several other genes [2].

Our data revealed high levels of gene expression for *DCN* and *COL2α1* in all samples of PC costal cartilage compared to an age-matched control. The male prevalence of these disorders was investigated in relation to the following X-linked cartilaginous related genes: *NYX, TIMP-1, BGN,* and *CACNA1F.* The *BGN* SLRP did not exhibit significant differences in either patients or controls. The *TIMP-1* gene was expressed with significant differences between patients and the age-matched control. The gene

expression ratios of *COL2α1/COL1α1*, *ACAN/COL1α1*, and *COL2α1/ACAN* were also lower than published values reported for articular chondrocytes, fibrocartilage of intervertebral discs and rat chondrosarcomas. However, in our study, many ratios were established for the first time in this type of PC tissue. There were observed changes in patterns of gene expression of costal cartilage from the control samples age, 23 weeks to 81 years old, indicating the importance of age-matched controls.

For future studies, our data can be compared to similar experiments on the "true ribs" and how the costal cartilage of these ribs may change in terms of gene expression with age, and which gene expression changes are important for adolescents with PC and or PE. Also, an investigation of X-linked gene expression in female cartilage is needed to better understand the etiology of PC or PE in relation to X-inactivation. Better understanding of the biology of these disorders will help in identifying causes and sequelae, leading to modified therapeutic options, particularly for those patients who do not respond well to chest wall surgical repair. This study provides new baseline patterns of gene expression in the context of the biology of costal cartilage and is a major contribution in the cartilage biology field.

This thesis is dedicated to the patients and their families of those who suffer from pectus disorders as well as their physicians, in hopes that my research may one day help these families and physicians better understand the biology of chest wall deformities and further the cartilage biology field.

ACKNOWLEDGMENTS

The completion of my graduate degree would not have been possible without the encouragement of my advisors and the opportunities they provided. I would like to acknowledge Dr. Christopher Osgood, my primary academic advisor, and Dr. Ralph Stevens, my undergraduate professor and mentor whom I taught Anatomy & Physiology laboratory under. I consistently appreciated the outstanding guidance from Dr. Michael Stacey and his wisdom and patience while I finished my research and Thesis. I would also like to thank my other committee member, Dr. Emilia Oleszak for her guidance, expertise, encouragement, and excitement for learning. I am also thankful for the time I was able to spend shadowing pectus surgeons, who studied under Dr. Nuss, in the clinic at CHKD. That experience provided great insight into the importance of this study. Finally, I extend my thanks to the entire staff of the Frank Reidy Center for Bioelectrics and the Department of Biological Sciences for their faculty's wisdom, training, patience, and assistance in the pursuit of my graduate degree.

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INTRODUCTION

Cartilage Biology

The human true and false ribs are connected to the sternum via a key feature of connective tissue: costal cartilage comprised of hyaline cartilage. The structure of hyaline cartilage contains chondrocytes, lacunae and an extracellular matrix (ECM) consisting of water, small and large proteoglycans, and other matrix proteins such as collagens that combine for support by forming fibers, (Figure 1). Collagen fibrils may run the length of hyaline cartilage within the ECM; however, their orientation is dependent on the forces they experience. The extracellular matrix of hyaline cartilage consists of proteoglycan monomers linked to hyaluronic acid filaments to form proteoglycan aggregates, aggrecan for example. The proteoglycan monomers are linked to a core protein via other link proteins. There is also a charge associated with extracellular matrix when chondroitin sulphate, a glycosaminoglycan, binds to the core protein. Ions and water molecules then become attracted, where the repulsion between the side chains creates distance between the proteoglycan monomers. Hyaline cartilage develops from mesenchymal progenitor stem cells *in utero* to become the cartilage forming cells, chondroblasts. These cells begin to secrete the ECM within chondrification centers, further separating the cells into isogenous groups where the cells can differentiate into chondrocytes, Figure 2.

Role of SLRPs

Of the small proteoglycans in costal cartilage, small leucine rich proteoglycans (SLRPs) regulate the fibrillogenesis and add strength to cartilage by cross-linking matrix proteins. Mutations in SLRPs have been shown to cause abnormal cartilage growth of the collagen fibers in mice [5]. The fact that some SLRPs also bind to the TGF-β protein to release growth factors, may contribute to the regulation of ECM development and chondrocyte proliferation. The growth factor gene, *TGF-β* and its downstream components may be abnormally-expressed in PC patient samples due to abnormal growth patterns exhibited on the costal cartilage of patients with PC [6].

The journal model for this thesis is *Connective Tissue Research.*

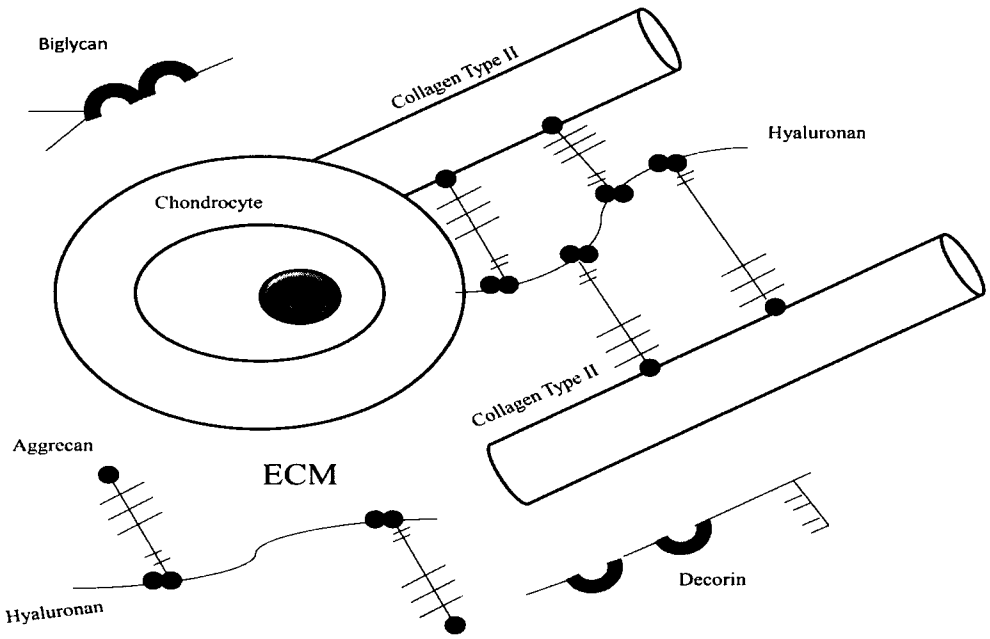

Figure 1. Interactions of cartilage extracellular matrix contents. [3]

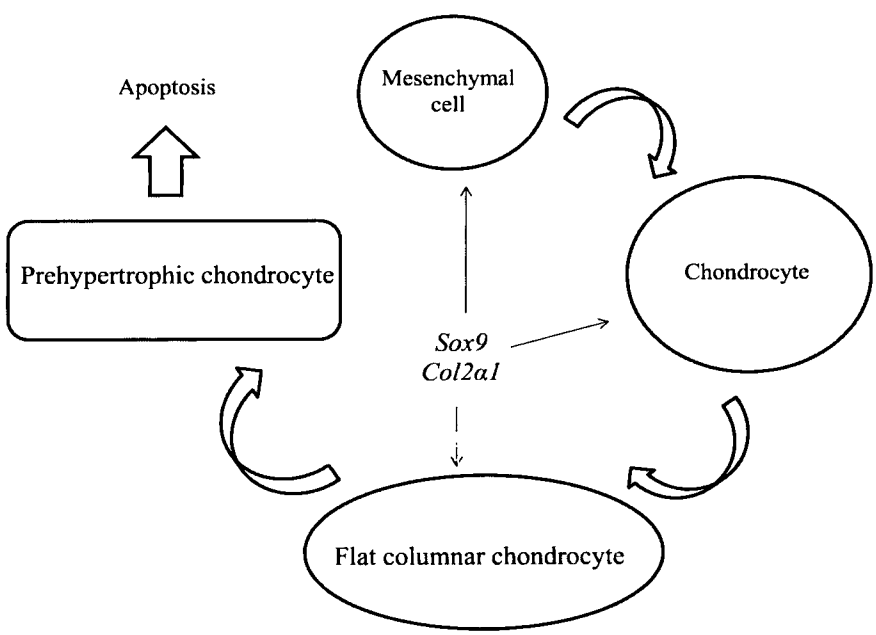

Figure 2. The early stages of chondrogenesis. [4]

Two specific SLRPs in our study were *BGN* (biglycan) and *DCN* (decorin). Interestingly, biglycan deficiency has been found to be a genetic characteristic of Marfan syndrome which is a connective tissue disorder often associated with PE that affects the growth of long bones, connective tissues, the heart, and eyes [7]. Decorin regulation is connected to the fibrillogenesis process [8, 9]. These SLRPs tend to compensate in expression for one another. For example, Zhang *et al.* investigated the interaction of these two SLRPs, decorin and biglycan, in relation to fibrillogenesis of the cornea in 2009. The group examined the functional roles of these two SLRPs in mice deficient for both genes and found that while decorin was expressed throughout development, biglycan is expressed early on at high levels and then decreased. They also discovered an up-regulation of biglycan in decorin deficient mice, but no up-regulation of decorin in biglycan deficient mice. Their findings indicated a cooperative interaction of biglycan and decorin in the formation of collagen fibrils within the cornea, and that decorin also plays a role in regulation of those fibers. [10]

Skeletal Dysplasias

Skeletal dysplasias affect approximately 1 in 2,000 people and include a mixed group of disorders. Chest wall deformities have been studied in relation to the presence of other skeletal dysplasias such as scoliosis, Poland's, Marfan's, and Ehlers-Danlos syndromes. Specifically, the altered weak connective tissue surrounding the chest cavity and spine may be linked to patterns of gene expression in costal cartilage from PC or PE patients [11]. The other skeletal dysplasias are also characterized by aberrant patterns of gene expression, for example: Marfan syndrome is a connective tissue disorder of the *FBN1* gene on chromosome 15 [11]. A mutation in the fibrillin protein of the *FBN1* gene prevents the correct formation of fibers of connective tissue that leads to a weakening of tissues in the heart, eyes, circulatory system, lungs and skeleton [7]. Scoliosis is another inherited disorder that is either autosomal dominant, X-linked, multigenic, or multifactorial and results in a curvature of the spine via deformation of the fibrocartilage located within intervertebral discs [12]. Ehler-Danlos syndrome is characterized by hypermobility of joints and "loose skin" that may be acquired via the mutation of the *COL5a1* or *COL5a2* genes that encode type V collagen as well as the genes encoding for the two forms of collagen type I fibers, *COL1a1* and *COL1a2* [13]. Several of these disorders have been

linked to Osteoporosis, another type of skeletal dysplasia that causes increased fragility in bone and decreased bone density in Marfan syndrome, Ehlers-Danlos syndrome, and scoliosis.

Chestwall Deformities

Pectus Disorders

Our research interest relates to cartilage biology in relation to chest wall deformities or pectus disorders. Chest wall abnormalities may be caused by a deformity of the ECM contents leading to a loss in function or change in structure of the costal cartilage [14]. There is a lack of blood flow through costal cartilage, which makes repair more difficult if degenerated or deformed, as in the case with PC (Figure 3) and PE (Figure 4) costal cartilage. Previous research on cartilage has focused on fibrocartilage, elastic cartilage, and synovial joint cartilage deformities. Hyaline cartilage involved in chest wall deformities differs from elastic cartilage and fibrocartilage in form and function: elastic cartilage has more elastin and elastic fibers in the ECM, functioning in more elastic tissue such as the epiglottis and pinna of the ear. Fibrocartilage composes the symphasis pubis and intervertebral discs, is purposed for support, and more rigid than elastic cartilage. This type of hyaline cartilage may be different than other hyaline cartilage found throughout the body such as in the trachea, airways, and joints. This study investigates patterns of gene expression from articular hyaline cartilage near the costochondral junction in patients with PC and those without this disorder.

Figure 3. Anatomical view of pectus carinatum. Informed consent from CHKD, form in Appendix, Figure 11. [16]

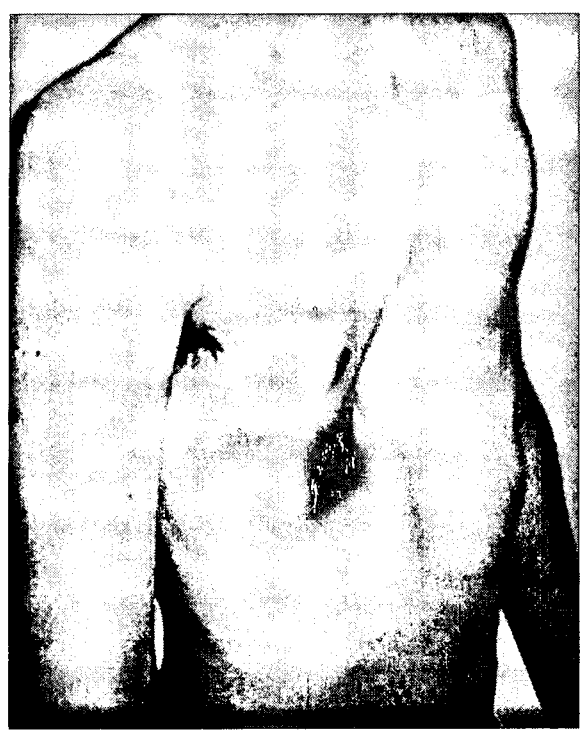

Figure 4. Anatomical views of pectus excavatum. [17]

Complex Inheritance

PC and PE are inherited connective tissue disorders with possible mutations in genes that code for the proteins that make up the ECM of the costal cartilage. The incidence of chest wall deformities of patients examined for chest wall deformities by Goretsky *et al.* was higher for PE (84%) than PC (15%) [11]. However, both disorders can severely affect the functions of the heart and lungs and can be very detrimental to self-esteem, especially in adolescence.

Pectus Corrective Surgeries

The treatment options for PE and PC differ based on the symmetry, caving or protrusion of the sternum. There are two main types of corrective surgery for PE: the Nuss and Ravitch procedures. The Nuss procedure was established in the 1980s as a minimally invasive corrective surgery contrasting the previously practiced more invasive Ravitch procedure. The Nuss procedure, invented by Dr. Donald Nuss of CHKD, makes two incisions on either side of the chest and inserts a curved steel bar which is then flipped and fixed to the ribs on either side. The bar normally remains in place for approximately two years and then is removed in outpatient surgery. The Ravitch procedure involves a horizontal cut across the chest and cutting of the costal cartilage to replace the sternum in a flat position. Often a bar is placed under the sternum to keep it in place. The treatment options for PC involve the reverse Nuss procedure or a dynamic compression brace, if PC is milder.

In the 1970s, surgeons practiced PE corrective surgery on children ages 4-6 using a modified Ravitch procedure where the chest cavity is opened so the costal cartilage can be cut on either side and the sternum flattened [18]. A bar is also inserted to stabilize the sternum and drains inserted into the affected area, followed by stitches to close the chest. Haller *et al.* investigated 254 children who underwent modified Ravitch repair of the chest deformity at Johns Hopkins Hospital. A new measurement of severity of the deformity was the caliper measurement in more than 50 children. Based on the postoperative results, this was the standard operation for correcting PE. [19]

The next step in PE surgery after the modified Ravitch procedure was to establish a more accurate means of measuring the degree of the chest deformity. To accomplish

this, Haller *et al.* used CT scans of the chest to evaluate the diameter of the chest compared to the anterior-posterior diameter. If the transverse diameter divided by the anterior-posterior diameter was greater than 3.25, this was the "pectus index" used to mark those who required surgery from those who did not (index <3.25). [20]

Management of both open and Nuss procedures has been both optional and required forms of surgical repairs. Kelly *et al.* found that surgical repair of PE improves patient's body image while reducing physical limitations of activity in 247 patients post operation, thereby improving the overall quality of life [15]. Kelly *et al.* performed a study to observe 327 patients enrolled to manage physiologic and pulmonary function as well as body image pre- and post-operation for PE at 11 centers in North America. They found that the abnormal pulmonary function was associated with those PE patients who had more severe cases and that the surgeries were performed safely, keeping perioperative complications and pain to a minimum.

Bioelectrical Properties of Cartilage

The electrical properties of cartilage may also contribute to the phenotypic and molecular changes of human costal cartilage in patients with PE and PC. Specifically, chondrocyte cell membranes must adapt to influx and efflux of ions in response to movement and changes in the ECM [14]. In biochemical studies described by Feng *et al.*, disturbance of costal cartilage type 2, seen in PE patients, was found to correlate to the abnormal levels of ions [14]. The passage of sodium, hydrogen and calcium ions across the chondrocyte membranes impacts the stability of the cartilage as the cells have to respond correctly to the movement [21].The external stimuli from the ECM, near the chondrocytes, converts an electrical potential when the ions move across the cell membranes. The dysfunction of these membranes may contribute to the phenotypic changes and increased weakness of the costal cartilage. One of the genes of interest in this study is *CACNA1F,* an X-linked gene that codes for a voltage dependent calcium channel (L type, alpha 1F subunit) [22].

Chondrocytes must be able to respond to the changing environment of the costal cartilage due to movement. The production of ECM from the cells is related to their ability to respond to pressure from their environment leading to normal or abnormal

functions of such tissues. In patients with PC or PE, it is possible that the cartilage is "weak" due to the chondrocyte response to stimuli. In this sense, chondrocytes may have an important role in the cartilage turnover involved in the strength of costal cartilage. The outer membranes of the chondrocytes have been suggested by Sanchez *et al.* to have altered ion transport and differences in electrical charge across the membrane [23]. Rollin *et al.* report that proteins can regulate ion transport across chondrocyte cell membranes such as those studied from osteoarthritis in relation to the TGFβ protein [24]. Much is known about the biological importance of ion transport across membranes in other tissue types such as the heart, muscles, and nerves. Cartilage is not recognized as an electrical tissue, but because of its environment, it can be considered an electrically responsive tissue type [6].

Costal cartilage can also become calcified with age if water content decreases and cavities form to calcify the matrix. The water content in costal cartilage is vital to its stability (Figure 5). The collagen fibers running through the ECM connect to chondrocytes and regions of larger proteins (proteoglycan monomers) that can attach to the fibers through link proteins. The attached proteins, like aggrecan, have a large protein core that other glycosaminoglycans attach to, such as chondroitin sulphate. The proteoglycan monomers attract water as an anion thereby contributing to the osmotic pressure of the costal cartilage. This tissue type then becomes negatively charged overall. Sun *et al.* described these fixed negative charges as the source of all electrochemical events in cartilage. [25]

Genes of Importance in Chondrogenesis

Gene interactions between chondrocytes of the costal cartilage involve a complex interaction of cell signaling and negative or positive feedback loops as seen in Figure 6, displaying a few of the genes of interest pertaining to this study. The 10 genes of interest for this study are listed in Table 1 and are comprised of a variety based upon their relation to cartilage properties, function, and chromosome linkage.

This study investigates key genes related to cartilage growth and turnover, including the *TGF-β* gene. The *TGF-β* gene cascade signals the activation of *SOX9* genes and positively up-regulates the *TIMP1* gene, inhibiting matrix metalloproteinases MMP8

and MMP13. Chondrocyte sensitivity to this growth factor can trigger the *COL2a1*as well as *SOX9* genes. Growth of human cartilage was shown to have increased *COL2a1* and aggrecan (*ACAN)* expression in differentiated chondrocytes; whereas undifferentiated cells had higher levels of *COL1a1* expression [26]. Aggrecan is the most abundant and largest proteoglycan present in the ECM of hyaline cartilage followed by decorin, biglycan, and fibromodulin [27]. By measuring the fold differences in gene expression of PC patients versus controls, we expect a difference in gene expression and gene expression ratios.

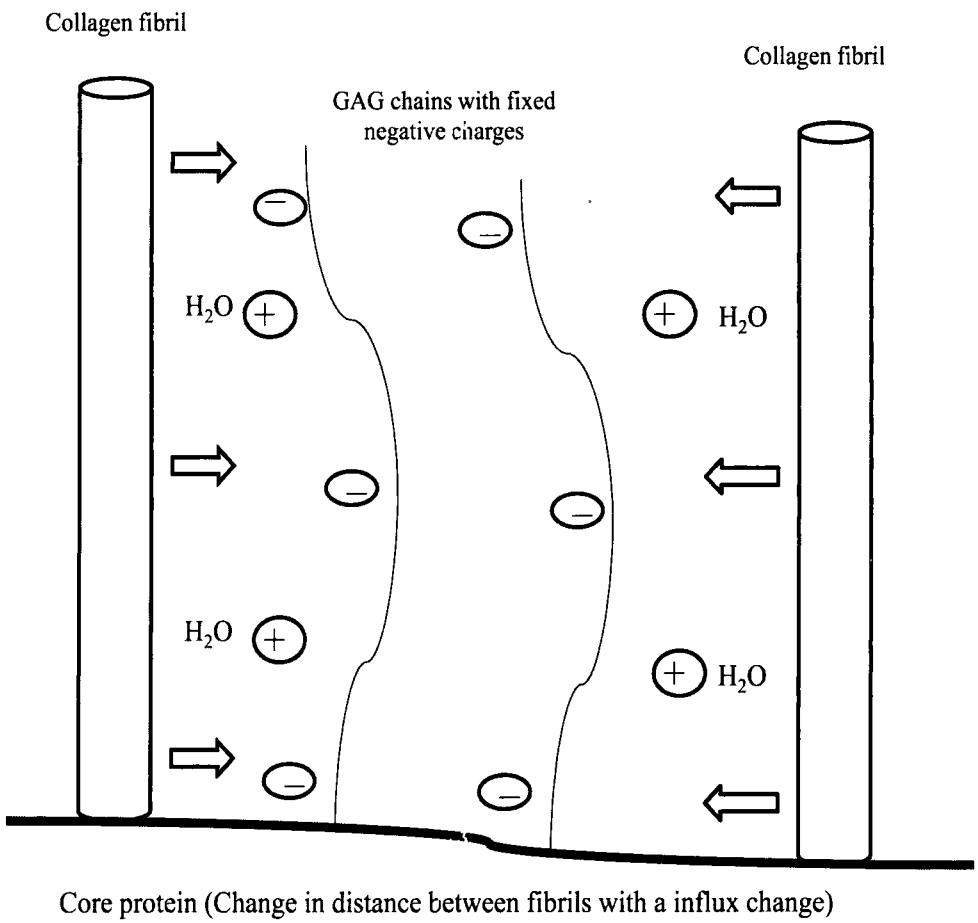

Figure 5. The water and ion content of cartilage. [28]

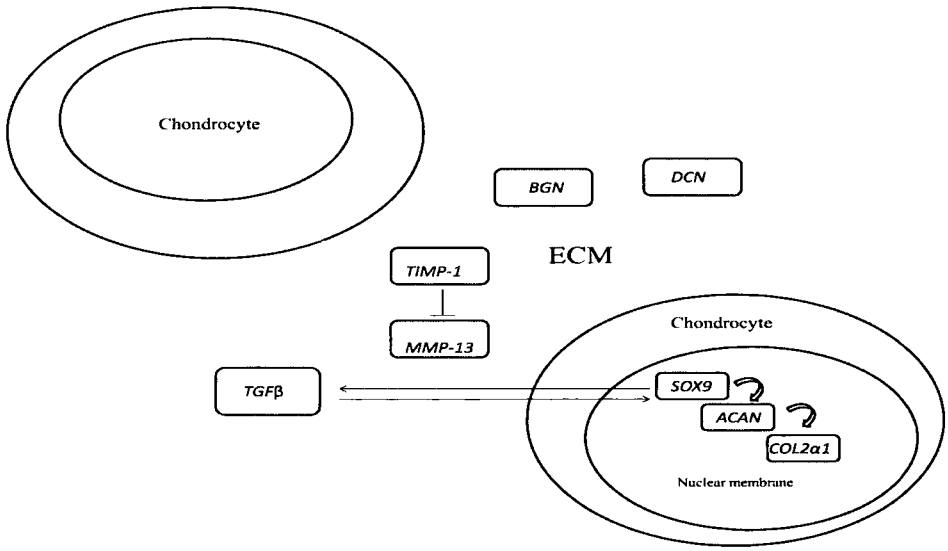

Figure 6. Gene signaling between chondrocytes. [29]

Aggrecan

Mutations of the *ACAN* gene are thought to cause skeletal dysplasia and thus the gene was investigated in correlation to PC. Regions on the *ACAN* gene were investigated for variable number tandem repeats along the CS1 domain of costal cartilage from PE patients based on the Haller index in a study by Dr. Stacey *et al.* [26]. Because of the role VNTRs play in attachment sites of chondroitin sulfate, it was hypothesized that abnormal numbers outside of the 26-28 range would correlate their prevalence to severity of PE. Of the females examined, 16% had increased severity of PE and fewer VNTR attachment sites, and therefore weaker cartilage. Although this study was performed on PE patients, *ACAN* seems to play a role in cartilage integrity involved in chest wall deformities, and is worth investigation in our study of PC samples. [26]

Biglycan

BGN is a small leucine rich proteoglycan on the X chromosome that encodes for the protein involved in assembly of collagen fibrils within the extracellular matrix of cartilage. [30]

Voltage-gated calcium channel-α1F

CACNA1F is an X-linked gene that encodes for a voltage-gated calcium channel protein. It functions to control the amount of calcium that enters the cell upon membrane polarization and may be linked to bioelectric components of cartilage with the passage of calcium ions across the cell membranes. [30]

Collagen α-1 chain

COL1A1 encodes for Type I collagen fibers found in most connective tissues. Mutations of this gene have been linked to Ehlers-Danlos syndrome, a disorder of connective tissue. [30]

Collagen type II α-1

COL2A1 encodes for collagen Type II fibers found in cartilage where mutations in this gene have been associated with chondrodysplasia and osteoarthritis. [30]

Decorin

DCN is another gene encoding for a small leucine rich proteoglycan related to the structure of the protein encoded for by *BGN* and functions to bind to collagen type I fibrils for matrix assembly. *DCN* has also been linked to Marfan syndrome, another disorder of connective tissue. [30]

Nyctalopin

The *NYX* gene was investigated because of its location on the X chromosome and its function as the gene encoding for a small leucine rich proteoglycan. [30]

SRY-box 9

SOX9 functions as a transcription regulator for skeletal development and plays a role in chondrogenesis and the other genes involved in that process by upregulating *TIMP-1*. [30]

Transforming Growth Factor-β1

TGFβ1 is a gene encoding for growth factor proteins that regulate proliferation and differentiation of many growth factors. [30]

Tissue inhibitor of metalloproteinase 1

The last gene of interest to be investigated is *TIMP1,* which is located on the X chromosome and functions as an inhibitor of matrix metalloproteinases (MMPs),

specifically MMP-8 and MMP-13, which are both collagenases. It also plays a role in the maintenance and turnover of the extracellular matrix within costal cartilage. [30]

β-Actin

The housekeeping gene used was *ACTB* (β-Actin), a highly conserved gene that encodes an isoform of actin in humans and is one of the non-muscle cytoskeletal actins. Actin is an important component in cell mobility, maintenance of cell structure, and cell integrity upkeep. [30]

Objectives and Hypotheses

This study aims to compare the genetic expression of PC cartilage samples to controls that do not have any known genetic association to these other connective tissue disorders in order to establish an accurate baseline level of gene expression. Specifically, the objectives of this study were to one: determine a baseline level for patterns of gene expression of key cartilage forming genes (*COL1α1, COL2α2, SOX9, ACAN,* and *DCN*) in costal cartilage of controls in order to identify changes in gene expression with age as well as identify changes between an age-matched control and patients with the chest wall deformity, pectus carinatum. And two: to determine levels of gene expression of four X-linked genes (*BGN, NYX, TIMP-1,* and *CACNA1F)* involved in cartilage synthesis and to examine if the single copies of genes from the X-chromosome are expressed or not in male patients with chest wall deformities versus an age-matched control. We hypothesized that (1) abnormal growth of costal cartilage in patients with a chest wall deformity will have different patterns of expression of key cartilage forming genes compared to control cartilage; and (2) due to the strong sex-linked component of chest wall deformities (males to females 4:1), we hypothesized that connective tissue related genes located on the X-chromosome will have different expression in males with a chest wall deformity compared to control tissues [15].

METHODS

Subjects

Because PC commonly requires surgical repairs to remove cartilage from ribs 6-8, costal cartilage samples were therefore gathered from these ribs: a combination of those that are attached to the sternum and those that are not. PC patient samples available were from males in the age range of teens to early twenties who agreed to donate samples after informed consent. Control cartilage samples were obtained based on available samples of those who recently deceased from Eastern Virginia Medical School (EVMS) or died of unrelated injuries. As with our patient samples, surgery typically takes place mid-late teens to early twenties. This complicates the process of obtaining normal, healthy age-matched control tissues and therefore control samples are limited. Samples were snap frozen in liquid nitrogen and stored at -80°C until use. Samples were taken from a 23 week old male (Control 1), an 81year old female (Control 2), and a 15 year old male as our age-matched sample (Control 3). After the genes of interest were selected for this study, RNA was extracted, reverse transcribed to cDNA, and amplified using real time PCR with SYBR green fluorescence as follows.

Research Compliances

Samples of human costal cartilage were obtained from consenting patients with PC who underwent surgical repair at the Children's Hospital of the King's Daughters (CHKD). Informed consent was received following Institutional Review Board (IRB) approval of the protocol by EVMS (#05-01-Ex-0027) and ODU (#12-093) as well as Institutional Biosafety Committee approval at ODU (#13-008) for research involving recombinant DNA, biohazards, and bloodborne pathogens training. Figure 11 (Form 0825 revisied 7/10) in the Appendix displays the release form patients signed to disclose images for use in scientific publications and presentations. There was total compliance with the appropriate federal and institutional guidelines for this study.

RNA Extraction

Control and patient samples of human costal cartilage (~ 0.2g cut into 2-3 mm chunks) were separately homogenized using a power tissue homogenizer (Fisher

Scientific Power Gen 35) in 2mL tubes using TRIzol Reagent. Some samples were also homogenized via pestle and mortar, under liquid nitrogen, until finely ground into a powder and then mixed with 2mL TRIzol Reagent per tube. TRIzol was used because of its ability to lyse cells and dissolve components while maintaining the integrity of the RNA. Chloroform (0.2mL of per mL TRIzol) was added to the tube of homogenate containing cartilage plus TRIzol and shaken for 20 seconds to separate the substances into an aqueous and organic phase. After centrifugation (eppendorf 5417R) at 10,000g for 18 minutes at 4°C or until an aqueous layer forms, RNA was isolated from the aqueous layer using the RNeasy® Plus Mini Kit following manufacture guidelines. RNA samples were then re-dissolved in 30µl of RNase-free water and spun at room temperature for 1 min at 8,000xg to elute the RNA. The OD concentration and $A_{260/280}$ readings were taken using (eppendorf BioPhotometer plus) or a spectrophotometer (GE Nanovue) to determine the purity of the RNA.

Reverse Transcription

Purified RNA samples (concentration of at least 5ng/µl) were reverse transcribed to cDNA via the RT^2 First Strand Kit followed by real-time PCR. 8 µl of extracted RNA was mixed with 2ul of GE (5x gDNA Elimination Buffer) and incubated in a water bath at 42°C for five minutes to eliminate contaminating genomic DNA from RNA samples, then chilled on ice for one minute to stop the reaction. 10 µl of RT Cocktail (4 µl buffer BC3, 1 µl control P2, 2 µl RE3 reverse transcriptase mix, 3µl H_20) was added to 10ul of Genomic DNA Elimination mixture and incubated at 42°C for 15 minutes then the reaction stopped by denaturing enzymes at 95°C for 5 minutes. DNA H_2O (91µl) was added to the cDNA synthesis reaction followed by concentration and $A_{260/280}$ readings taken using (eppendorf BioPhotometer plus) to determine the purity and yield of cDNA. The average total yield per reaction was 220 ng/µl of cDNA.

Real-Time PCR Analysis

PCR reactions were performed via the Qiagen RT-PCR protocol using the BioRad CFX96 system. The PCR master mix included 5µg/µl of cDNA, 6.5 µl DNA water, and 12.5 µl SYBR green for a total of 25µl per well (including 1 µl of the primer) and was analyzed in duplicate or triplicate using SYBR green detection (Qiagen, CA USA). After

the initial denaturation step at 95°C for 10 min, the cDNA products were amplified with 40 PCR cycles consisting of a denaturation step at 95°C for 15 s and an extension step at 60°C for one min. A melting curve from 65°C to 95°C in 0.2°C increments for five minutes determined the specificity of the reaction.

Data Interpretation

Data analysis for each cDNA sample was carried out using the BioRad CFX Manager™ software for which Ct values (cycle number across a designated threshold) of the target gene were subtracted from the Ct housekeeping gene, *ACT*-β values [32]. Fold differences 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 [33].

Statistical Analysis

Statistical analysis was performed using the Student t-test to determine significant differences between samples and control averages. For all of the trials, a p-value < 0.05 was indicative of statistically significant differences. For every sample there were 4-6 extractions to yield RNA that could be reverse transcribed to cDNA. For each gene of interest examined per sample, the genes were run in duplicates or triplicate. Only those with consistent results across the sample and genes were used for analysis.

RESULTS

Genes of Interest

In the field of cartilage biology, there is a gap in understanding of the genetic and molecular aspects that contribute to chest wall deformities such as PC and PE. Our study initially investigated the genetic expression of PC abnormalities in comparison to normal costal cartilage based on gene expression. The most important aspect to our data collection was the quality of our RNA and cDNA yields from each tissue extraction. This procedure was difficult at first to define; however, we established that for RNA, a concentration of at least 5ng/μl was needed to proceed to the reverse transcription step. The purity of the RNA obtained ($A_{260/280}$ values) ranged from 1.01 to 1.78. Generally, the concentration and purity of the RNA isolated was less than that of the cDNA (220 ng/μl yield per reaction).

Table 1 displays genomic locations of the genes of interest studied and the house keeping gene, β-Actin, a positive control. The chromosome location of the gene was important to the investigation of sex-linked genes related to cartilage formation because earlier reports had shown a male prevalence ratio of 4:1 for the incidence of PC [1]. These genes of interest have key roles in relation to cartilage turnover and development of chondrocytes within their extracellular matrix and were examined for their relation to PC cartilage.

Melting Temperatures and Curves

The accuracy of the RT-PCR was evidenced by the results of the melt-curve and melt-peak analysis: where genes had relatively similar melting temperatures across many trials. An example of accurate experimental melt-peak data can be seen in Figure 7 and melt-curve data in Figure 8. The melt peak and curve data are displayed for *BGN*, *DCN*, *TIMP1*, and *TGF-β1* as examples; each run completed in duplicate. Melt peak temperatures are important because they denote primer specificity for hybridization, which can theoretically be used to detect mutations between alleles. Melt curve data are measured for the temperature at which 50% of the DNA has denatured based on the drop in fluorescence. Where the genomic double stranded DNA reached its melting

temperature and denatured to become single stranded, a drop in fluorescence of SYBR green was recorded as relative fluorescence units (RFU) vs. temperature °C, (Figure 7 and 8, Table 2). Experimental temperatures of both samples were the same. The melting temperatures measured were also very consistent across the samples data (PCs and controls) and repeatable between experiments, indicating no genetic mutations of the genes analyzed by PCR. If there were peaks with lower melting temperatures, the differing peaks could indicate primer-dimer artifacts, contamination, or mispriming. [34]

Table 1. Genes of interest in this study, their chromosome locations, and descriptions of function. [6, 30]

Gene	Name	Chromosome Location	Description
ACTβ	β-Actin	7p22	Housekeeping
ACAN	Aggrecan	15q26.1	Large aggregating proteoglycan
BGN	Biglycan	Xq28	Small proteoglycan (SLRP)
CACNα1F	Voltage-gated calcium channel-α1F	Xp11.23	Voltage-sensitive calcium channel
COL1α1	Collagen α-1 chain	17q21.33	Type I collagen fiber
COL2α1	Collagen type II α-1	12q13.11	Type II collagen fiber
DCN	Decorin	12q21.33	Small proteoglycan (SLRP)
NYX	Nyctalopin	Xp11.4	Small proteoglycan (SLRP)
SOX9	SRY (Sex determining region Y)-box 9	17q24.3	Transcription regulator
TGFβ1	Transforming Growth Factor-β1	19q13.2	Growth factor
TIMP-1	Tissue inhibitor of metalloproteinase 1	Xp11.23	Inhibitor of matrix metalloproteinases (MMPs)

Our comparison of both theoretical and experimental melt peak temperatures of genes of interest is found in Table 2, where standard deviations between samples and genes ranged from 0.00-0.91. Software for high resolution melt curve analysis is currently unavailable in our laboratory. The mean experimental melt peak for *COL2A1* fell in the range of the theoretical melt peak temperature, whereas *COL1A1* resulted in a three degree difference from the theoretical. This was also the case for *BGN,* which resulted in a 2.8- 4.8°C decrease in experimental melting temperature compared to the theoretical temperature. *ACAN* was about one degree lower experimentally (83.4°C instead of 84.2°C). *TIMP1* differed from the theoretical by 1.9°C. *DCN* only differed by 0.1-0.9°C. Three of the genes did not have melting temperatures recorded: *SOX9* because data were gathered before the set-up of melting temperatures, while *NYX* and *CACNA1F* did not result in measurable amplification.

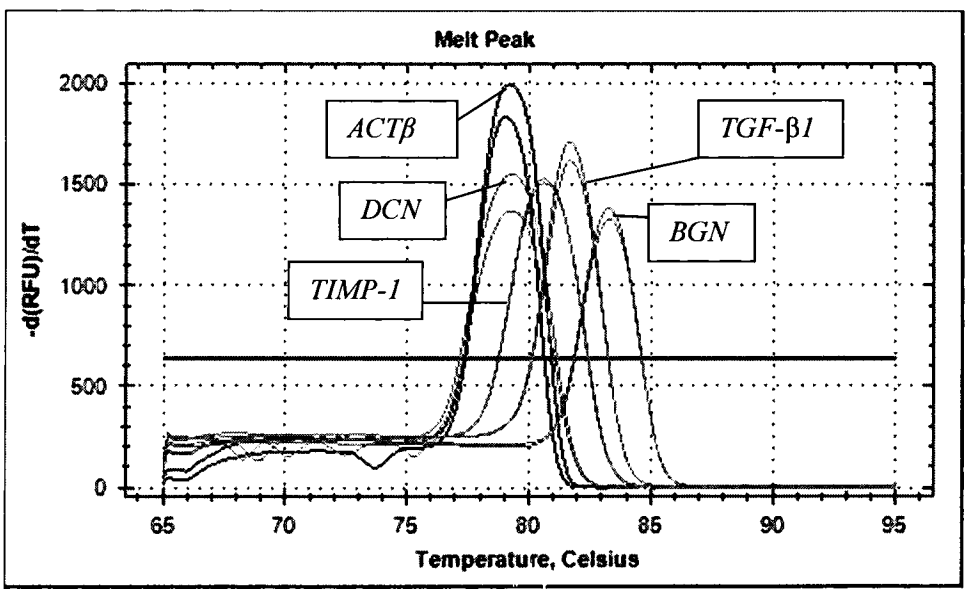

Figure 7. Melting peak temperatures of genes of interest.

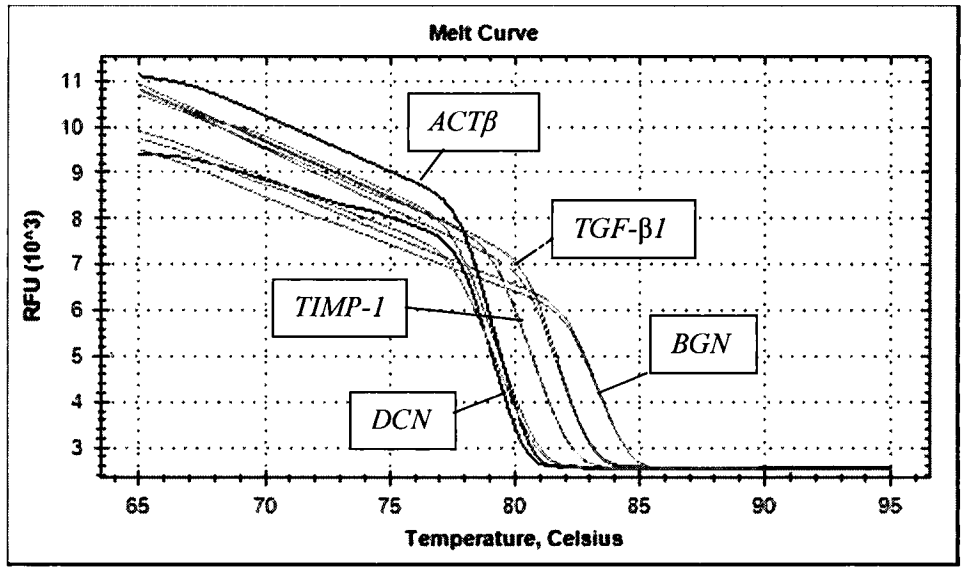

Figure 8. Melting curve temperatures of genes of interest.

Gene Expression Comparisons

Real time PCR was performed on RNA extracted from cartilage from both patients and controls. Relative values of gene expression were made to the positive control, β-actin (*ACT* β), using the Ct method. Figure 9 displays the results of PCR amplification of *BGN, TIMP1, DCN* and *TGF-β1,* where high gene expression is indicated by the lowest number of cycles and the fastest increase in cDNA replication across the threshold bar (~10^2 RFU). High gene expression is therefore displayed as a curve farthest to the left of *ACTβ*, while the curves amplifying at higher cycle number, and on the right, have lower expression. In Figure 9, *DCN* had the higher gene expression compared to *ACTβ* with the lowest Ct value. *TIMP1, BGN,* and *TGF-β1* had increasingly higher Ct values, but lower gene expression compared to *ACTβ.*

Gene amplification was determined compared to three controls that represented extremes of age differences (Control 1 and Control 2) and an age-matched control (Control 3) as seen in Table 3. Note that normal costal cartilage is difficult to obtain in age-matched controls as chest wall deformity repair tends to be between the ages 15-25 years, and so only one sample was used in this study.

Table 2. Sample melting temperatures for theoretical melt temperatures (italicized) vs. experimental melt temperatures. *BGN,* Biglycan; *TIMP1*, Tissue inhibitor of metalloproteinase 1; *DCN*, Decorin; *TGFβ1,* Transforming Growth Factor- beta 1; COL2α1, Collagen type II α-1; COL1α1, Collagen α-1 chain; *ACAN,* Aggrecan; *SOX9,* Sex determining region Y-box 9; and ND, not determined. **information not available.

	Mean Experimental vs. Theoretical Melting Temperature							
Theoretical Melt Temp. (°C)	*BGN* *85.8*	*DCN* *79.9*	*TIMP1* *81.9*	*TGF-β1* **	*COL2α1* *80.9*	*COL1α1* *83*	*ACAN* *84.2*	*SOX9* **
Sample:								
PC1	83.2	79.3	80.6	81.8	ND	ND	ND	ND
PC2	83.2	79.4	80.6	81.8	ND	ND	ND	ND
	83.1	79.2	80.5	81.5				
	83.2	79.3	80.5	81.7				
	83.0	79.2	80.5	81.4				
	83.2	79.3	80.6	81.6				
PC3	83.2	79.0	80.5	81.7	ND	ND	ND	ND
PC4	82.8	79.3	80.4	81.8	ND	ND	ND	ND
	83.0	79.1	80.3	81.7				
Con1	82.7	79.3	80.5	81.8	ND	80.8	83.4	85.6
	83.2	79.4	80.8	81.8				
Con2		79.3	80.5	81.8	ND	ND	ND	ND
		79.0	80.5					
		78.9						
Con3	83.2	79.2	80.6	81.6	ND	ND	ND	ND
	83.2	79.2	80.6	81.6	79.5	80.6	83.4	85.5
		79.2	80.3	81.4	80.8	80.8	83.4	85.6
Mean	83.1	79.2	80.5	81.7	80.2	80.7	83.4	85.6
St.dev.	0.17	0.14	0.12	0.14	0.91	0.11	0.000	0.05

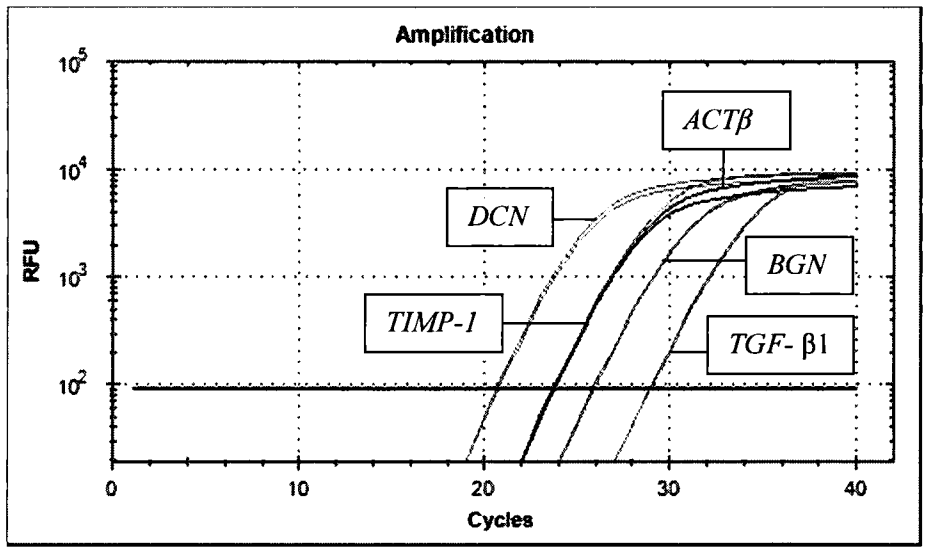

Figure 9. Gene expression curves. High expression is displayed as a curve farthest to the left of the to the house keeping gene *ACTβ*, while those curves closer have lower expression.

Fold differences in gene expression in control samples

Table 3 shows the mean fold differences of gene expression for the control samples normalized to *ACT* β. Values greater than 1.0 indicate gene expression more than *ACTβ* and are highlighted

Control 1, a 23-week-old male fetus, showed high levels of expression of *COL2α1, DCN, TIMP1,* and *COL1α1*compared to actin (208.4x, 13.36x, 6.51x, and 4.29x respectively). The higher expression for *COL1α1* ($p<0.001$) and lower expression of *ACAN* ($p<0.003$) observed with Control 1 is a likely reflection of the immature development and partial differentiation of the costal cartilage at such a young age.

Control 2, the 81 year old female, had the highest expression of *DCN* at 25.91x than that of *ACT* β. There was also significant reduction of *COL2α1* ($p<0.0001$) and *ACAN* ($p<0.003$) expression in this sample, a likely product of old age.

Table 3. Mean fold differences of gene expression (+/- SE) in three control patients compared to expression of to the house keeping gene *ACTβ*. Shaded areas indicate higher expression, those >1. 00. [6]

Gene	Control 1	Control 2	Control 3
COL2α1	208.4+/-40.76	0.009+/-0.004	134.0+/-7.06
ACAN	0.108+/-0.019	0.198+/-0.031	3.12+/-0.69
DCN	13.36+/-8.68	25.91+/-6.12	17.63+/-2.25
TIMP-1	6.514+/-5.82	3.69+/-0.71	3.18+/-0.47
BGN	0.516+/-0.133	0.033+/-0.012	1.39+/-0.49
COL1α1	4.29+/-0.73	0.109+/-0.025	0.12+/-0.03
SOX9	0.007+/-0.001	0.041+/-0.013	0.327+/-0.105
TGF-β1	0.118+/-0.036	0.157+/-0.046	0.179+/-0.094

Control 3, a 15 year old male, was used as an example of cartilage that is close to maturation. It resulted in five genes expressed higher than the *ACTβ* house keeping gene where the results of the gene expression were *COL2α1* (134.0x), *ACAN* (3.12x), *DCN* (17.63), *TIMP-1* (3.18), and *BGN* (1.39). The difference in expression of *DCN* and *BGN* was not significant in this sample ($p<0.23$ and $p<0.07$ respectively). For the rest of this study, Control 3 was used for comparison to the other PC samples because of the proximity in age.

In comparison between Control 1 and Control 2, there were no significant difference in expression of *DCN* and *BGN* ($p<0.54$ and $p<0.2$ respectively). However, the overall results in control samples show expression of genes that are associated with cartilage and fibril organization, including *COL2A1, ACAN,* and *DCN.* The level of *TIMP1* expression, the highly regulated gene that controls collagen turnover by MMP8 and MMP13, was similar across the control patient samples (no significant differences between Control 3 and Control 1 ($p<0.42$) or Control 2 ($p<0.56$)). Other genes expressed

less than *COL2A1, ACAN,* and *DCN*, include the transcription factors *SOX9* and *TGFβ.* An apparent age-dependent expression of certain genes associated with development, maturation, and maintenance of control costal cartilages was observed.

Fold differences in gene expression in patient samples

Comparisons of the patterns of gene expression in costal cartilage from four patients with PC to that of the age-matched control showed the following: the highest expression (highlighted if greater than 1.0) occurred in *COL2α1, ACAN, DCN, TIMP1,* and *BGN* compared to the housekeeping gene *ACT*β (Table 4). Table 4 displays patterns of variable gene expression from four PC patients compared to one age-matched control where significant differences were those with a p value < 0.05. Of the five highly expressed genes, *COL2α1* had the highest expression in all samples. PC1 showed significant reduction in expression of *DCN* ($p<0.001$) and *TIMP1* ($p<0.001$) compared to the control sample. PC2 had decreased expression of *COL2A1* ($p<0.01$), *DCN* ($p<0.0002$), *TIMP1* ($p<0.001$), and *BGN* ($p<0.03$). PC3 showed lower expression of *COL2A1* ($p<0.001$) than the control with decreased expression of *ACAN* in PC3 ($p<0.03$) and increased expression of *ACAN* in PC4 ($p<0.024$). PC4 had increased expression of *TIMP1* ($p<0.001$), but decreased expression of *BGN* ($p<0.04$) in that sample. The two other X-linked genes of study, *NYX* and *CACNA1F,* did not have expression in any of the samples. The variable gene expression levels between patient samples vs. the control sample may be due to the age range within the cases or the handling of the cartilage post-surgery.

Table 4. Fold difference in gene expression (+/- SE) of four patients with PC and an age-matched control compared to *ACT*β. Significant differences ($p<0.05$) in expression between control and patients are marked with an *. Shaded areas indicate higher expression, those >1. 00. [6]

Gene	Control 3	PC1	PC2	PC3	PC4
COL2α1	134.0+/-7.06	134.31+/-49.33	*84.09+/-10.42	*46.96+/-12.22	118.79+/-10.24
ACAN	3.12+/-0.69	3.79+/-1.4	2.89+/-0.26	*1.30+/-0.15	*6.38+/-0.989
DCN	17.63+/-2.25	*4.47+/-0.51	*4.31+/-0.71	16.21+/-0.71	20.58+/-2.18
TIMP-1	3.18+/-0.47	*0.67+/-0.07	*0.91+/-0.159	3.39+/-0.01	*5.53+/-0.46
*ACT*β	1	1	1	1	1
BGN	1.39+/-0.49	0.59+/-0.07	*0.33+/-0.09	0.84+/-0.01	*0.3+/-0.054
COL1α1	0.12+/-0.03	0.15+/-0.03	0.12+/-0.02	0.11+/-0.04	0.12+/-0.02
SOX9	0.33+/-0.10	0.30+/-0.17	0.20+/-0.02	0.15+/-0.001	0.63+/-0.098
TGF-β1	0.18+/-0.09	0.15+/-0.021	0.13+/-0.041	0.09+/-0.016	0.04+/-0.005

Cartilage Gene Expression Ratios – a measure of differentiation

Gene expression ratios have been used to estimate cartilage differentiation. For example, the ratio of *COL2α1:COL1α1* decreases dramatically as cartilage cells de-differentiate. From the gene expression data compiled in this study, gene ratios were calculated to investigate differentiation in costal cartilage (Table 5). We expected to see higher expression of *COL2α1* in differentiated cartilage with higher expression of *COL1α1* in more undifferentiated cartilage. The ratios were first compared to published data for articular cartilage and fibrocartilage of lumbar discs [31]. Rabbit articular chondrocytes had a published ratio of *COL2α1:ACAN* of 1,090; whereas, our data ranged from 18.62-36.12 for the PC patients and 42.95 in the age-matched control [31]. The rabbit articular chondrocytes ratio of *COL2α1:COL1α1* was 1,790 [31], while our data ranged from 426.9-989.9 in PC samples and 1116.7 for the age-matched control. The nucleus pulposus region of lumbar discs of rabbits have published expression ratios for *COL2α1:ACAN* and *COL2α1:COL1α1* of 23 and 930, respectively, comparable to our results [31]. In rat chondrosarcoma cells, the ratios of *ACAN:COL1α1* are around 78.4,

similar to our data (11.82-53.17 for PC samples and 26 for Control 3) [31]. Our data also support this ratio published for dedifferentiated chondrocytes cultured from costal cartilage at 4.6 [31]. From studies on human articular cartilage, published ratios of *COL2α1:COL1α1* have been reported as 294.6, but these results were compared to *GAPDH* as the housekeeping gene, not *ACTβ*. [31]

The differences discovered in gene expression ratios between PC patients and an age-matched control appear small and are likely not a major contribution to chest wall abnormalities in this sample studied (Table 5). The largest difference was observed between age groups, suggesting a change in expression with age. In our study, the data showed higher expression of *DCN* compared to *BGN* (7.58-69.06 in PC samples and 12.68 in Control 3) suggesting a role of *DCN* expression in costal cartilage morphology. Decorin is known to be present at high levels during the development of tendons and in this case, may parallel the expression in costal cartilage development (hyaline cartilage) because of the similarity in collagen fibers to those fibers in tendons [24].

Table 5. Gene expression ratios as markers of differentiation in costal cartilage. Control 3 is the age-matched control for comparison to the other four PC samples. NP, nucleus pulposus of lumbar disks; Acs, articular chondrocytes; Rcs, rat chondrosarcoma cells.

Sample	*COL2α1/ ACAN*	*COL2α1/ COL1α 1*	*ACAN/ COL1α1*	*DCN/ BGN*	*COL2α1/ TGF-β1*	*ACAN/ SOX9*
PC1[6]	35.44	877.8	24.77	7.58	895.4	12.63
PC2 [6]	29.1	700.75	24.08	13.06	637.05	14.45
PC3 [6]	36.12	426.91	11.82	19.34	539.77	8.97
PC4 [6]	18.62	989.92	53.17	69.06	3210.54	10.08
Con3 [6]	42.95	1116.7	26	12.68	748.6	9.54
Con1 [6]	1929.63	48.58	0.025	25.89	1766.1	15.43
Con2 [6]	0.045	0.08	1.82	785.15	0.057	4.83
NP [31]	23	930	4.6 *in vitro*			
AF [31]	370	26				
Acs [31]	1090	1790				
Rcs [31]			78.4			

DISCUSSION

The etiology of PE is unknown and is the most common congenital deformity of the chest wall present between 1 in 400 and 1 in 1000 live births. As of 2006, Creswick *et al.* [1] performed a pedigree analysis of 34 families to investigate whether PE is a inherited, familial disorder. In total, 14 families suggested autosomal dominant inheritance, 4 suggested autosomal recessive inheritance, 6 families suggest X-linked recessive inheritance, and the remaining 10 families had complex patterns of inheritance. These results further confirm the genetic complexity of the chest wall deformity, pectus exavatum. [1]

Sugiura was one of the first to describe PE, then termed funnel chest. In 1977 his team in Japan studied 6,295 school children (age 6-12) of which eight children were diagnosed with varying degrees of funnel chest. After a pedigree analysis, they found that in one family, four individuals from three generations were affected and indicated an autosomal dominant inheritance to this disorder. [35]

The previous research leading up to this study provided insight into the possible male prevalence of chest wall deformities, where a pedigree study by Horth *et al.* suggested a role of expression of cartilaginous related genes on the X chromosome [36]. Leung and Hoo, in 2005, published their research on three Chinese families in which congenital "funnel chest" or PE was segregating [37]. There study also confirmed that PE can be transmitted as a dominant trait: equally transmitted by men or women, does not skip a generation, and each offspring has a 50% chance of inheriting the mutation. Genetic traits, such as PE and PC are expressed due to loci on chromosomes X or Y that are dominant or recessive. Because males have only one X-chromosome allele, normally expression is likely. [37]

The genetic expression patterns in hyaline cartilage from costal cartilage of PC patients are not well understood, yet this information would be a key aspect of understanding cartilage biology in the chest wall. Costal cartilage is made up mainly of collagen type II and the most common proteoglycan such as aggregan, followed by biglycan and decorin. Results of interest were the expression levels of the two SLRPs: decorin and biglycan; as well as the other large proteoglycan, aggrecan; and the expected

proteins in cartilage: COL2α1 and COL1α1. Collagen fibrils of the ECM interact with proteoglycans, such as aggrecan, through their glycosaminoglycan side chains. Vynios *et al.* described interactions between type I collagen and decorin due to ionic strength further contributing to the maintenance and integrity of this tissue. [38]

Decorin and biglycan were first described in 1989, where Fisher *et al.* established two forms of small proteoglycans of bone: one containing only one chondroitin sulfate chain and the other with two. They termed the one with two chains PG I and the other PG II and correlated their presence in many types of connective tissues. Fisher *et al.* suggested PG I be called biglycan because of its two chains and the other be called decorin. [39]

Fisher *et al.* also credited decorin and biglycan with playing critical roles in the maintenance of the ECM in hyaline cartilage when they described the non-collagenous proteins of organic bone matrix. Through the use of antisera and cDNA probes to human bone matrix non-collagenous proteins, they discovered that many of the proteins critical to formation of bone matrix may not be found in the final matrix product. They suggested the protein's role was in synthesis and then diffusion into the blood stream. [40]

Decorin's 3D protein structure was described in 1996 by Weber *et al.* in relation to collagen fibrillogenesis across four species, including human. The conformation of the protein is arched at the center and has ten leucine-rich repeats with only one glycosaminoglycan chain and three oligosaccharides linked to the *N*-terminus. The shape of the center domain allows the protein to attach to one collagen triple helix by fitting tightly after the addition of a second collagen molecule and regulate the arrangement of fibrils. [41]

As of January 2010, decorin was investigated for its role in modulating the formation of collagen fibers of the ECM in relation to the TGFβ superfamily and bone morphogenetic protein-1 (BMP-1) [42]. There are two states of decorin described here: the pro and mature forms. Fisher *et al.* described their study on three isoforms of BMP-1 that are able to cleave the propeptide from prodecorin, resulting in the mature proteoglycan. The addition of decorin's glycosaminoglycan chain was not inhibited by

the removal of the propeptide. This type of prodecorin has been used clinically to reduce the amount of fibrosis experienced in organs such as the kidney and heart. [42]

The localization of decorin and biglycan in human intervertebral discs (IVDs) containing fibrocartilage was investigated in three regions (nucleus pulposus, annulus fibrosus, and end plates) [31]. These two proteoglycans were found by Götz *et al.* to function mostly as mechanical and structural support for IVDs in the ECM by regulating fluid flow and charge distribution in the discs. Decorin and biglycan were found in the outermost areas of the annulus fibrosus, thereby contributing to matrix formation of collagen and roles in stability. In the innermost regions of the IVD, the nucleus pulpsus region had decorin labeled antibodies within the interterritorial matrix, thereby contributing to binding of fibrils containing collagen type II. Biglycan was found in the extracellular matrix on the nucleus pulposus region. For the endplate region, results were similar to that of articular hyaline cartilage: decorin antibody mostly labeled the interterritorial matrix, while the biglycan antibody labeled the cytoplasm of the chonodrocytes of the end plates. With immunoreactivity studies on age, the IVDs showed decreases in both decorin and biglycan, also similar to that of human articular cartilage. [43]

Young *et al.* found that biglycan has an essential role in the regulation of chondrogenesis and ECM turnover in relation to temporomandibular joint osteoarthritis. This joint consists of type I and type II collagen where modifications to biglycan in the ECM can lead to osteoarthritis in that joint. *BGN* binds to *TGF-β1*, the growth factor responsible for the formation and degradation of the ECM and the maintenance of cartilage. Their study investigated how the ECM regulates differentiation and function of mandibular condylar chondrocytes and found that the absence of the BGN protein led to an overall imbalance of the ECM turnover, favoring cartilage degradation. [44]

Our other gene of interest pertained to the transcription regulator, *Sox9* (sex determining region Y-box 9). Smits *et al.* described how *Sox6* is expressed in cartilage along with *Sox5* to enhance chondroblast functions and the formation of the endochondral skeleton. This process occurs in the chest wall with the formation of the sternum and ribs connected to the costal cartilage at the costal notches and costochondral junctions. The

formation of mature chondrocytes occurs via differentiation from chondroprogenitor cells of embryogenesis where they migrate to different locations in the body and differentiate into prechondrocytes. These cells then aggregate and activate cartilage gene markers to differentiate into cells that can create cartilage where they can be referred to as chondroblasts and are able to produce the ECM. This differentiation is controlled by *Sox9* and *Cbfa1*, where it is related to the activation of *Col2a1*, a cartilage specific enhancer. [45]

It is still not well understood how isolated chest wall deformities occur. However, several connective tissue disorders and syndromes with causative agents are associated with representation of PE or PC, i.e. Marfan or Noonan syndromes. The gene mutated in Marfan syndrome is *Fibrillin-1* from chromosome 15 in which PE or PC are hallmarks of this syndrome. Noonan syndrome is an autosomal dominant disorder involving RasMAPK (mitogen activated protein kinase) pathways. Because of such close associations of PC or PE with other genetic abnormalities, patients should be examined for chromosomal aberrations. [46]

The clinical development of PC or PE throughout adolescence may largely be related to the environment of the extracellular material surrounding the chondrocytes, which directs their differentiation. In later stages of differentiation, the matrix gets remodeled due to the activation of aggrecan and collagen II. Hormones also play a role in chondrocyte maturation including thyroid hormones, insulin, or insulin-growth factors where cartilage of the ribs is vascularized post natal, but not mineralized until after adolescence and furthermore in the third and fourth decade of life [47]. According to Bahramia, the endochondral ossification processes of costal cartilage arrests after adolescence and after chondrocytes reach their hypertrophic stage of late differentiation. This stage is marked by two markers: collagen X and alkaline phosphatase, which increase in the period of adolescence. Ultimately, the etiology of chest wall deformities is very complex and may have other external contributions aside from inheritance patterns. [47]

This study established a baseline reference for patterns of gene expression of key cartilaginous forming genes in normal and PC costal cartilage samples e.g., those without

other chondral dysplasias. Specifically, we investigated the relative gene expression of major genes involved in the growth of cartilage from different ages (23wk-81yr) and clinical conditions (control and PC samples) to better understand how costal cartilage is maintained and how it differentiates.

Our main objectives were to identify changes in gene expression with age as well as changes between an age-matched control and patients with the chest wall deformity, PC. We focused on patterns of gene expression of key cartilage forming genes normalized to *ACTβ* (*COL1α1, COL2α2, SOX9, ACAN,* and *DCN)* and four X-linked genes (*BGN, NYX, TIMP-1,* and *CACNA1F).*

Melting curves and peaks have been used by Hentosh *et al.* and others as methods to measure the accuracy of primer specificity and the presence of possible mutations with the use of our genes of interest. The results for melting curves and melting peaks for our genes of interest were accurate across samples and within the same gene of interest with very minimal standard deviations. Comparing the theoretical and experimental melting temperatures for the genes, there were no significant differences for most of the genes, except for *BGN* and *COL1α1*, which deferred from the theoretical temperature by 2.7°C. A possible explanation would be a malfunction of the PCR machine, or degradation of the primer. Overall, our data were consistent: primers annealed properly and specific genes were amplified correctly. Melting peaks were consistent and repeatable, indicating no genetic mutations of the genes run on PCR. Specifically, the peaks for each gene run on PCR displayed similar peaks under one another per sample, a likely indication of few to no primer-dimer artifacts, contamination, or mispriming present in our results. [34]

The Ct method provided an accurate means to measure relative gene expression and mean fold differences of gene expression for each gene comparing PC samples versus an age-matched control from gene expression curves as described in a study by Livak and Pombo-Suarez [48]. Like Suarez's results that established reference genes for qPCR of cartilage from hip and knee joints, our data established *ACTβ* as a suitable reference gene for gene expression tests of costal cartilage. The use of SYBR green fluorescence in real-time PCR and melt curve analysis provided a means of distinguishing normal amplification and those with possible mutations. Melting peak

analysis-compatible software could be used in the future to better analyze this data as Hentosh *et al.* performed in their examination of molecular approaches to detect genetic mutations and variants. [34]

The three controls had a range of ages, so the age-matched control (Control 3) was chosen for the continuation of our analysis against the four PC samples, which varied in age from mid-teens to early twenties [Table 4]. Control 3 had the most patterns of expression above the house keeping gene of our chosen genes of interest. Because our study investigated adolescent age PC samples, it was thought best to match our control in age. Higher expression occurred in all PC samples, but only for *COL2α1, ACAN, DCN, TIMP-1,* and *BGN* genes. Patterns of gene expression occurred between our age-matched control and PC samples with significant differences in: *DCN* for PC1 and PC2; *TIMP-1* expression for PC1, PC2, and PC4; *COL2α1* for PC2 and PC3; *ACAN* in PC3 and PC4; and *BGN* for PC2. The down regulation of *TIMP-1* in PC1 and PC2, but up regulation in PC3 and PC4 compared to the age-matched control indicated an interesting change in this gene expression between samples. This down regulation suggests influences on fibrillogenesis and matrix turnover in the costal cartilage and is a likely reflection of heterogeneity. However, *COL1α1* was under expressed in both the age-matched control and the PC samples examined, but was consistent across all samples. PC1 had the closest patterns of gene expression compared to our age-matched control, differing in *DCN, TIMP-1,* and *BGN* expression. It is difficult to rule out degradation of PC samples post operation or degradation of the RNA and cDNA obtained from the sample. PC is however a complex disorder with a complex genetic pattern made up of many genes, some of which may influence the phenotypic development of the costal cartilage of PC patients. The clinical details of the PC patients were not obtained for this study, but in the future, may provide important insight into the genetic variability among PC samples [15].

Also, the increased expression of *DCN* in all PC samples, and under expression of *BGN* in PC samples indicates a possible inverse relationship like that seen in the study of IVDs by Götz *et al.* [43]. Biglycan's role in encoding for the protein involved in the assembly of collagen fibrils within the ECM of cartilage indicates that it was not doing so in the PC samples; whereas, decorin was expressed higher than the housekeeping gene

and functions to bind collagen type I fibrils for matrix assembly and stability [10]. For these SLRPs, our results detected high expression of *DCN,* versus *BGN,* and no expression of *NYX,* thereby possibly not involved in chondrogenesis of our samples. However, we were unable to distinguish between the pro and mature forms of *DCN* and *BGN* described by Fisher *et al.* [10,38,39]. The primers used for gene expression studies are not specific regarding their targets: pro or mature forms of the proteins. An investigation into the differentiation and localization of *DCN* forms involved in collagen fibrillogenesis is a topic for the next study. Although *DCN* plays a role in modulation cartilage fibril growth, thickness, and orientation, its over expression may be compensation for the under expression of *BGN* seen in our results. The size or thickness of the cartilage samples may have also contributed to the over expression of DCN. These levels of *BGN* and *DCN* expression change with age: where increased decorin and decreased biglycan reported are consistent with our finding.

Gene expression ratios have been used by others to determine the differentiation status of cartilage. We used gene expression ratios to estimate the level of cartilage differentiation of the PC samples compared to our age-matched control and other control tissues where *COL2a1* is highly expressed when chondrocytes differentiate. During dedifferentiation *in vitro*, *COL1a1* expression increases and *COL2a1* and *ACAN* decrease [31]. This was seen in the 81-yr-old with reduced presence of aggrecan and increased collagen type 1. Our data fill gaps in knowledge of what is known and reported already for the ratios of gene expression in costal cartilage of different ages: fetal, teen, and the elderly, but more samples would be needed for the future. Not much is known on gene expression of cartilage in fetal development, and the fetal samples are expected to be very different due to the environment in the amniotic sac. Compared to published values of gene expression ratios, our PC data most closely correlate to expression of the nucleus pulposus of lumbar discs than to articular cartilage or rat chondrosarcoma cells, an indication of functional requirements to their loads of the spine and chest [31].

In addition, several changes were made to experimental parameters in order to get the best yield of RNA and cDNA. Regarding the purity of RNA and cDNA extracted and reverse transcribed respectively, there were difficulties in this isolation. The high ratio of

matrix to cells decreased the A260/280 value due to ECM contents. Our methods section was adjusted in order to create the best density and OD readings. The extractions of cartilage were first doubled to begin the RNA isolation, performing two isolations at one time and then combining the aqueous layers above the thick protein layer after centrifugation in Trizol. The extraction step at least continued to the RT step in order to yield cDNA the same day the RNA was extracted and then frozen at -80°C until use. All PCR reactions were run in duplicate or triplicate in order to establish mean Ct values and to better display accuracy of our results. Finally, half-way through the project, a new RT kit was used to maintain the integrity of our experiment.

The results of this study met our hypotheses in that: (1) abnormal growth of costal cartilage in patients with PC had different patterns of expression of key cartilage forming genes as exemplified by the increased expression of *COL2α1, ACAN, DCN, TIMP-1,* and *BGN* genes, except for in PC1; (2) due to the strong sex-linked component of chest wall deformities (4:1), connective tissue related genes located on the X-chromosome had different expression in males with PC compared to controls with different ages where an age-matched control comparison was made. A likely factor of the expression in males is due to the fact that males have only one X-chromosome and express the genes on that chromosome, whereas females have two X-chromosomes and may or may not express the genes on one X-chromosome. In the future, more female PC samples are needed to make the results more conclusive.

Results were conclusive in high levels of *DCN* expression and *COL2α1/COL1α1* gene ratios indicating under differentiation. A proposed theory of these patterns of gene expression involves the role of SLRPs, which have an important role in fibrillogenesis of the collagen in the ECM and also regulate growth factors in order to shape the mechanical properties of the ECM. Because the PC samples were similar in gene expression ratios to that of the NP of intervertebral discs, the data suggest an important function of this cartilage is load bearing. The differences in gene ratios seem small and not major contributions to chest wall deformities such as PC; however, in this study, the largest differences between patterns of gene expression were between the age groups for control cartilage samples.

The results of this study will be of use to future studies on both PC and PE costal cartilage. Future experimentation, although more costly, should involve gene arrays to better understand the gene expression of PC or PE compared to controls. If possible, more samples of PC and PE cartilage would be ideal as well as age-matched controls without other skeletal dysplasias. In the future, we hope to compare our data to similar experiments on variations along and between different ribs to correlate altered functions within the chest wall. Because of the phenotypic variation between PC patients, genetic variation was expected; however, in the future more aged matched controls would be necessary. In this study, costal cartilage changed genetically with age and so we would like to investigate a more broad age range. Our results indicate how important it is to have an accurate age-matched control or multiple studies pertaining to PC or PE. To better understand the etiology of these disorders, further investigation is needed for the genetic expression on the X-chromosome in female cartilage in affiliation with X-inactivation. PC and PE may also be caused by external factors or inheritance, a cause that remains difficult to pinpoint due to the complex etiology of these deformities.

CONCLUSIONS

Our data indicate that costal cartilage demonstrates patterns of high levels of *COL2α1* and *DCN* expression in both PC samples and our age-matched control. Given the strong specificity of hybridization of the primers and the melting peaks lacking much variation between samples, our data are both precise and accurate. The experimental melting temperatures between samples for every gene of interest varied from no standard deviation to 0.91 °C. The mean experimental melting peaks, another way of examining the sensitivity of our PCR results, compared to theoretical melt peak temperature provided by the primer manufacturer varied from 0.7 – 2.7 °C. There were gene expression data collected that expressed two of the X-linked genes: *NYX* and *CACNA1F.* Although the *DCN* SLRP was highly expressed, the other SLRP of study, *BGN*, did not result in high levels of gene expression, except for in the age-matched control. The third highest expression in PC samples compared to our age-matched control tissue was *TIMP-1*, a gene responsible for cartilage turnover.

Considering the gene expression ratios, *COL2α1/COL1α1* resulted in patterns of gene expression lower than those observed in published ratios [10, 31]. Many other gene expression ratios were established in this study as a first contribution to this type of PC costal cartilage tissue. There was however, observed change in gene expression patterns of costal cartilage control tissues with time (23 weeks -81years old) indicating the importance of age-matched controls with this type of study. This study provides new baseline genetic characteristics about the biology of costal cartilage, a major contribution to the cartilage biology field.

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APPENDIX

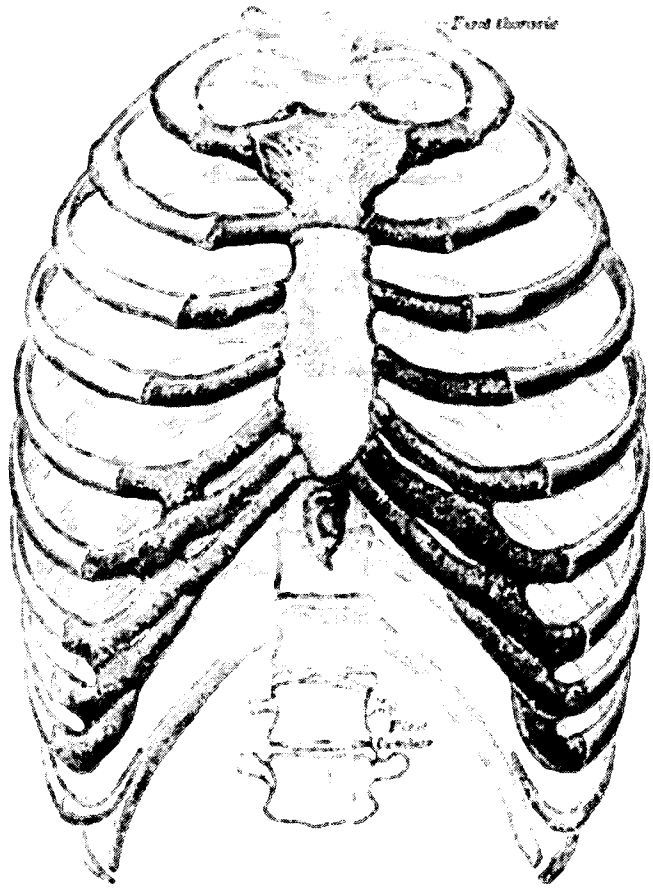
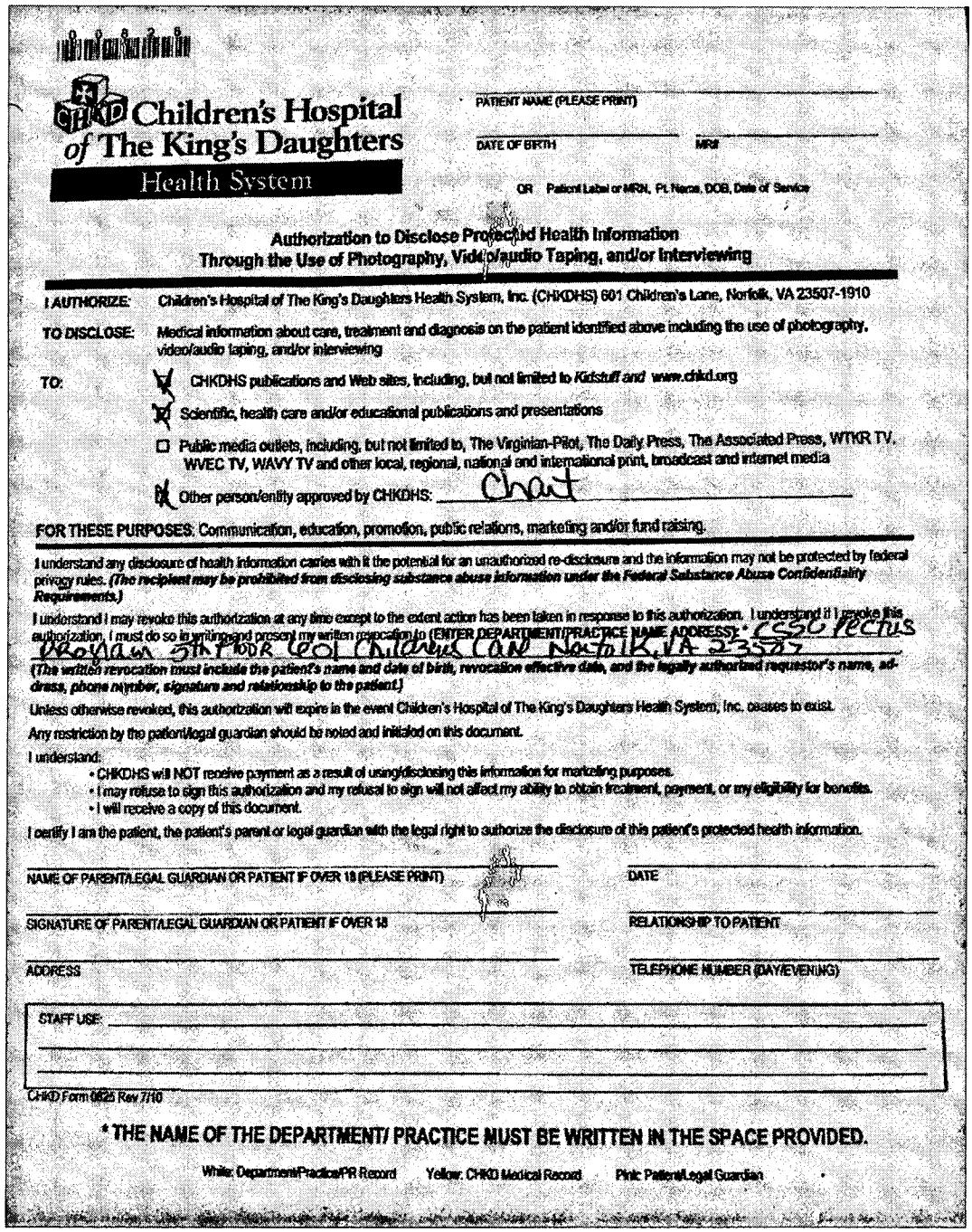


Figure 10. Thoracic cage of costal cartilage. [49]



CHKD Children's Hospital of The King's Daughters Health System

PATIENT NAME (PLEASE PRINT)

DATE OF BIRTH MR#

OR Patient Label or MRN, Pt Name, DOB, Date of Service

Authorization to Disclose Protected Health Information Through the Use of Photography, Video/audio Taping, and/or Interviewing

I AUTHORIZE: Children's Hospital of The King's Daughters Health System, Inc. (CHKDHS) 601 Children's Lane, Norfolk, VA 23507-1910

TO DISCLOSE: Medical information about care, treatment and diagnosis on the patient identified above including the use of photography, video/audio taping, and/or interviewing

TO:

- [X] CHKDHS publications and Web sites, including, but not limited to *Kidstuff and* www.chkd.org
- [X] Scientific, health care and/or educational publications and presentations
- [ ] Public media outlets, including, but not limited to, The Virginian-Pilot, The Daily Press, The Associated Press, WTKR TV, WVEC TV, WAVY TV and other local, regional, national and international print, broadcast and internet media
- [X] Other person/entity approved by CHKDHS: Chart

FOR THESE PURPOSES: Communication, education, promotion, public relations, marketing and/or fund raising.

I understand any disclosure of health information carries with it the potential for an unauthorized re-disclosure and the information may not be protected by federal privacy rules. ***(The recipient may be prohibited from disclosing substance abuse information under the Federal Substance Abuse Confidentiality Requirements.)***

I understand I may revoke this authorization at any time except to the extent action has been taken in response to this authorization. I understand if I revoke this authorization, I must do so in writing and present my written revocation to (ENTER DEPARTMENT/PRACTICE NAME, ADDRESS): * CSC Pectus Program 5th floor 601 Children's Lane Norfolk, VA 23507

(The written revocation must include the patient's name and date of birth, revocation effective date, and the legally authorized requestor's name, address, phone number, signature and relationship to the patient.)

Unless otherwise revoked, this authorization will expire in the event Children's Hospital of The King's Daughters Health System, Inc. ceases to exist.

Any restriction by the patient/legal guardian should be noted and initialed on this document.

I understand:

- CHKDHS will NOT receive payment as a result of using/disclosing this information for marketing purposes.
- I may refuse to sign this authorization and my refusal to sign will not affect my ability to obtain treatment, payment, or my eligibility for benefits.
- I will receive a copy of this document.

I certify I am the patient, the patient's parent or legal guardian with the legal right to authorize the disclosure of this patient's protected health information.

NAME OF PARENT/LEGAL GUARDIAN OR PATIENT IF OVER 18 (PLEASE PRINT) DATE

SIGNATURE OF PARENT/LEGAL GUARDIAN OR PATIENT IF OVER 18 RELATIONSHIP TO PATIENT

ADDRESS TELEPHONE NUMBER (DAY/EVENING)

STAFF USE:

CHKD Form 0625 Rev 7/10

*** THE NAME OF THE DEPARTMENT/ PRACTICE MUST BE WRITTEN IN THE SPACE PROVIDED.**

White: Department/Practice/PR Record Yellow: CHKD Medical Record Pink: Patient/Legal Guardian

Figure 11. IBC approval from CHKD.

VITA

Janna E. Grubbs

Department of Biological Sciences

Old Dominion University

Norfolk, VA. 23508

Education

Bachelor of Science in Biology

Old Dominion University, May 2010

Master of Science in Biology

Old Dominion University, May 2013

Teaching/Research Experience

- Graduate Teaching Assistant: Anatomy & Physiology 250/251
  May 2011-December 2012
  Department of Biology, Old Dominion University

- Graduate Teaching Assistant: Techniques of Mammalian Tissue Culture
  May 2011
  Department of Biology, Old Dominion University

- Research Technician: The Frank Reidy Center for Bioelectrics
  September 2011- May 2012
  Old Dominion University

Presentation

- Eastern Virginia Medical Center, Department of Pediatrics, August 2012, Norfolk, Virginia.

Publications

Stacey, M.W., Grubbs, J., Asmar, A., Pryor, J., Elsayed-Ali, H., Cao, W., Beskok, A., Dutta, D., Darby, D.A., Fecteau, A., Werner, A., Kelly, R.E. Jr. (2012). Decorin expression, straw-like structure, and differentiation of human costal cartilage. *Connective Tissue Research.* 53:415-21.

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