# Expression and localization of Connexin 43 in cartilage of horses with Osteochondrosis dissecans

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## Abstract

Osteochondrosis dissecans (OCD) in growing individuals represents a chondrodysplasia with uncertain etiology. Regions of retained cartilage result from retardation of the progression of subchondral ossification (Glaser et al). Focal avascular necrosis is hypothesized to initiate OCD. The molecular events defining OCD, a prevalent developmental joint disease in human and animal, have been limited to the study of a relatively small number of candidate molecules. After examining a large-scale microarray study, two of the most dysregulated genes were Connexin 43 (Cx43), a gap junction protein encoded by the GJAP1 gene, which provides routes for the movement of low-molecular weight materials, and Proteoglycan 4 (PRG4), a surface lubricating protein.

Using more advanced quantitative real-time polymerase chain reaction (RT-PCR) method, Cx43 was found to be expressed 7-fold higher in OCD cartilage than normal cartilage, and PRG4 was found to be expressed 3-times lower in OCD cartilage than normal cartilage. Through histological methods of immunohistochemistry and *in-situ* hybridization, Cx43's expression and translation were found to be around the area of osteoclasts/ chondroclasts as well as the subchondral bone separation as a result of the OCD lesion with clefting.

We hypothesize Cx43 has the ability to attenuate the inflammatory processes and further damage the surrounding tissues. At the same time, PRG4 is down-regulated due to the surrounding inflammatory environment.

## Acknowledgements

Although my name may be printed on the front page of this honors thesis, I could not have done it without the unwavering support from so many people. First and foremost, I would like to thank Dr. Nixon for giving me the opportunity of a lifetime working in the Comparative Orthopaedics Laboratory. Thank for introducing me to the world of orthopedics, sharing those Cuban cigars and rainmaking fireworks. You have opened so many doors for me. It is hard to express my gratitude in these simple words.

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# Introduction

## Cartilage and Its Growth

Cartilage is a type of dense connective tissue. It is composed of specialized cells called chondrocytes that produce a large amount of extracellular matrix composed of cartilage fibers, abundant ground substances rich in proteoglycan, and elastin fibers. Healthy articular cartilage is the tough but flexible tissue that protects the ends of bones and allows for smooth motion in joints. Unlike other connective tissues, cartilage does not contain blood vessels. Healthy cartilage allows bones to glide over each other. It also protects the bones by preventing them from rubbing each other. However, due to compressive stresses of daily living, cartilage is prone to tears and injuries, which may lead to chronic inflammation and tip the balance in favor of catabolic mechanisms. Inflamed or damaged cartilage can cause symptoms such as chronic pain and limited movement, considerably decreasing the quality of life.

There are two regions of growth cartilage that are present at the ends of long bones during the period of bone growth and development. In the growth of bone, growth cartilage is constantly replaced by bone through a sequential process of cell proliferation, extracellular matrix synthesis, cellular hypertrophy, matrix mineralization, and vascular invasion that is termed enchondral ossification [35]. The invasion of cartilage by blood

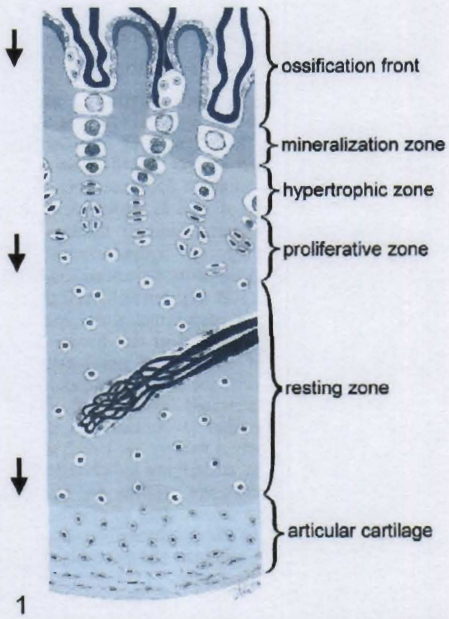


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**Fig. 1.** Schematic cross-section of an articular–epiphyseal cartilage complex including the terminal end of a cartilage canal. The cartilage canal contains an arteriole branching into numerous capillaries that rejoin into a venule. In the immature animal, the cells of the proliferative zone will continuously divide, and the cells of the hypertrophic zone will increase in size, causing the cartilage complex to grow in the direction of the arrows. On the ossification front, vess[35] d bone cells invade the cartilage in the same direct lecause the growth of the cartilage is slightly slower than the progression of the ossification front, the growth cartilage will become successively thinner.

vessels is required for enchondral ossification, as illustrated by the fact that genetic, biochemical, or mechanical disruption of angiogenesis results in thickening of the epiphyseal growth plate due to expansion of the hypertrophic zone. These networks of vessels course through the cartilage by ways of cartilage canals. However, the articular cartilage remains avascular. The purpose of the cartilage canals is still unclear. It has been suggested that they contribute to three purposes: to nourish chondrocytes beyond the reach of diffusive nutrients from the synovial fluid, to play a role in the formation of the secondary ossification center, and to supply both the cartilage and bone with mesenchymal stem cells.[35]

As the individual grows, the growth rate of the cartilage slows compared with the rate of the ossification front, causing the ossification front to advance towards the articular cartilage and resulting in the layer of growth cartilage to become thinner. By the time of adulthood, the epiphyseal growth cartilage is replaced by bone, and no cartilage canals remain.

**Ostechondrosis Dissecans (OCD)**

Ostechondrosis dissecans is a common and debilitating cartilage disease in growing animals and children, but can also be found among the adult population, especially among athletes and dancers. The condition is described as a pathologic condition in the articular cartilage, a condition that causes a predisposition to the formation of loose bodies in the joint space. The primary lesion involves detached sheets of cartilage and destabilizes the joint surface, resulting in pain, dysfunction, and increased risk of osteoarthritis.

Articular osteochondrosis (OC) is characterized histologically by a focal area of necrosis that is confined to the growth cartilage, and involves either the overlying articular cartilage or the underlying subchondral bone. OCD describes the condition after which a fissure forms in the area of necrotic cartilage that extends through the articular cartilage, creating a flap or loose body.

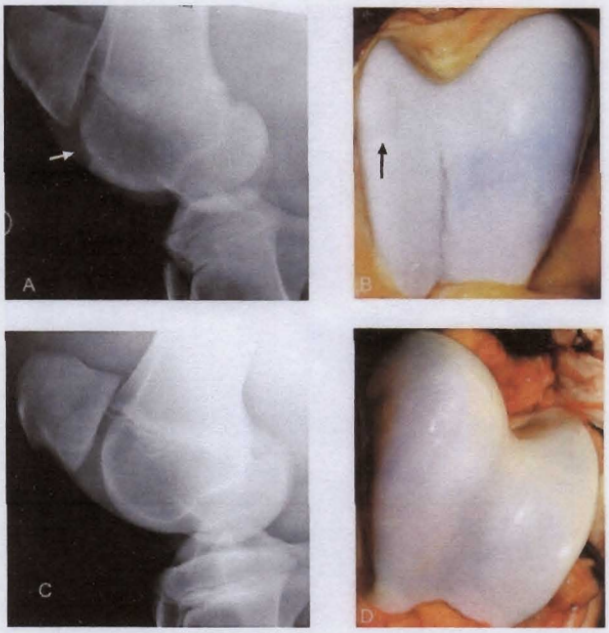



**Figure 2. (A, B) Radiographic and gross appearance of early OCD lesions of the lateral trochlear ridge of 7 months old foal. (C,D) Age matched normal radiograph and smooth cartilage surface. [10]**

In horses, osteochondrosis is one of the frequent causes of lameness in young athletic horses. In a radiographic survey of young Standardbred trotters, 14.3% were found to have OCD in the tibiotarsal joint.[12]

**Etiology**

The etiology has been intensively researched, but the progress has been hampered by the unclear pathogenesis, particularly regarding the formation of the primary lesion. This appears to be due to the fact that most researchers have focused their studies on lesions that cause clinical signs or are associated with radiographic lesions. At these chronic stages of disease, the information obtained usually reflects the secondary degenerative and regenerative processes, rather than the primary processes.[35]

Currently osteochondrosis is regarded to have multiple etiologies, with no single factor accounting for all aspects of the disease. Suggested factors that play a role in this disease include rapid growth, heredity, anatomic characteristics, trauma, dietary factors and a defect in vascular supply to epiphyseal cartilage.[35]

**Pathogenesis**

The leading theory for the pathogenesis of OC and OCD from the observation that areas of chondronecrosis are closely associated with necrotic, nonperfused vessels in cartilage channels. In experimental studies, interruption of cartilage canal blood supply rapidly causes necrosis of the surrounding cartilage, creating lesions typical of naturally occurring osteochondrosis. This finding suggests that naturally occurring osteochondrosis is the result of local events that causes failure to a limited number of cartilage canals. Ytrehus et al. proposed that the primary event in osteochondrosis is the cessation of cartilage canal blood supply that occurs as a result of damage to temporal and newly formed vessels.

**Figure 3. Cartilage samples. There is evidence of necrosis at the subchondral cartilage junction.**

**Current Treatments**

After ischemic necrosis of growth cartilage occurs, the overlying articular cartilage, being avascular, has limited ability to respond to injury. Lesions tend to progress in size and severity, affecting the rest of the articular cartilage and increasing the risk of osteoarthritis. Depending on the size and severity of the osteochondral defect, a technique known as retrograde drilling can be used to fill the lesion with

bone cement. Another technique known as microdrilling or micropicking can be used to stimulate the formation of fibrocartilage to replace the original cartilage. A procedure used for larger lesions is called Osteochondral Autologous Transfer (OATS), where a graft of bone and cartilage is taken from a different location and implanted to the site of the defect. Other techniques include polydioxanone (PDS) pinning or screw fixation, to stabilize the lesion back onto the subchondral bone. A more biological technique is autologous chondrocyte implantation, where the patient's own chondrocytes are harvested, cultured in vitro, and put back into the patient underneath a periosteal flap harvested from the patient as well.

However, the abovementioned surgical treatments very rarely return the cartilage to its original state. The best treatment of this disease is its prevention, but in order to do so, the etiology, molecular mechanism, and pathogenesis of OCD must first be better understood. To this point, only a small number of candidate molecules including growth factors, degrading enzymes, and matrix molecules have been studied in connection to OCD.

**The Current Study**

Although numerous cases of OCD can be explained by trauma to the joint, just as many cases cannot be explained by trauma. This points us to explore a more genetic and molecular basis to the disease. A previous study on equine OCD cartilage used differential display technology and microarray GeneChip ® studies to identify for the first time a large-scale altered gene expression in OCD lesions of horse.[25] Later, Glaser et al was able to more specifically identify the specific genes dysregulated in OCD lesions of the lateral trochlear ridge of the distal femur in 7 to 9

months old horses. Based on these findings, OCD can result from altered gene expression as well as a matrix structural change.

The molecular events of OCD by profiling gene dysregulation in the early stages of OCD using an equine large-scale (12,000 genes) oligonucleotide exon array (GeneChip ®, Affymetrix). After analysis, Connexin 43 (Cx43) was found to be one of the most up-regulated genes, and therefore a potential key player involved in the pathogenesis of early osteochondrosis lesions in horses.[25]

Cx43, encoded by the GJAP1 gene, is a member of the Connexin gene family that encodes for gap junction proteins which provide routes for the movement of low molecular weight materials from cell to cell, such as glucose, ions, and ATP.[3,13,17,27] Cx43's role has been predominately studied in the brain and heart; its role in cartilage is not completely understood.

Another altered gene in microarray studies was Proteoglycan 4 (PRG4), which has a rich history of names. It began as Superficial Zone Protein (SZP), which was then changed to Lubricin, and now it is primarily known as Proteoglycan 4. Its primary role is to act as a lubricating glycoprotein responsible for boundary lubrication in synovial joints. PRG4 is a component of synovial fluid that is synthesized by the superficial chondrocytes in both articular cartilage and synovial cells. A thin layer of PRG4 is present on the surface of articular cartilage to provide the necessary lubrication.

We hypothesize Cx43 over abundance is a relatively early molecular player in the pathogenesis of OCD. Necrosis and bleeding associated with OCD have the ability to release proinflammatory signals. These signals much travel from its origin

to surrounding tissues. Being a gap junction protein, it has the ability to transmit inflammatory cytokines and chemokines, such as TNF-α, which act directly on cells in the site and amplify the inflammatory response and further damage the surrounding tissues. PRG4 on the other hand is down-regulated due to its regulation by the surrounding inflammatory processes.

However, as we know, no full mRNA sequence of this gene in horses was available. In order to further investigate the molecular mechanisms of OCD, this study identified the sequences of the genes, and more closely examined the localization of the RNA and protein expression in OCD lesion by using quantitative real-time PCR, immunohistochemistry, and *in-situ* hybridization.

# Materials and Methods

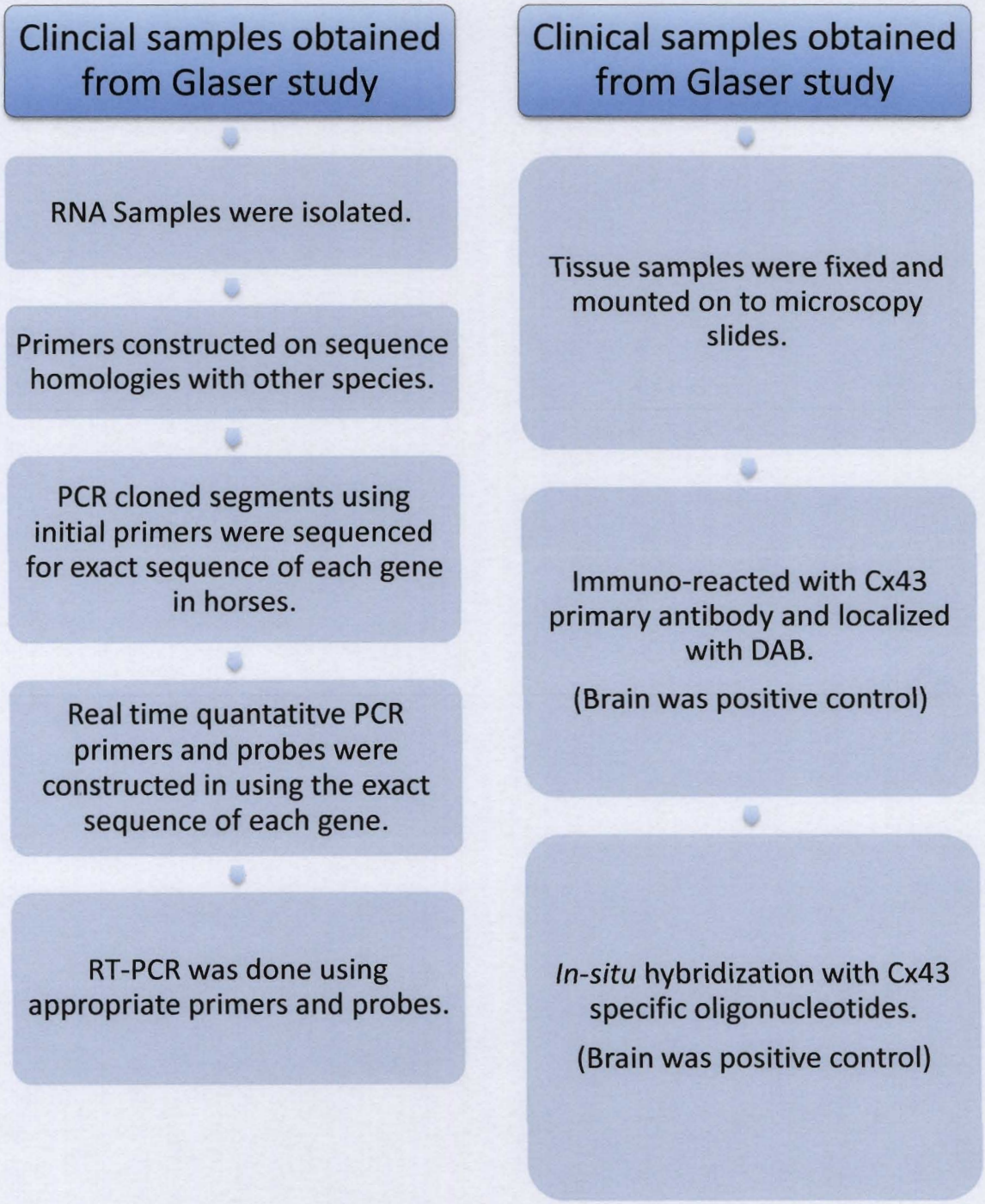
**Overview**

To examine the expression of Cx43 and PRG4 by quantitative PCR and *in-situ* hybridization, very specific DNA probes had to be designed from its sequence. In order to determine their sequences, Cx43 and PRG4 were cloned from tissues that express them in abundance, equine chondrocytes. The cloned genes were then reverse transcribed to yield its respective cDNA. The cDNA was then used as the template for polymerase chain reaction (PCR) to amplify each of the genes, which were then ligated into a TOPO vector. The vector was transfected into competent *E. coli*, then grown on LB plates. Positive growth colonies were subsequently grown in LB broth to allow adequate amplification of the vector for sequencing. The reported sequence was then entered to BLAST to compare the sequence against the initial release of the equine genome and to the sequence in other species.

Once the sequence was determined, a quantitative real-time PCR probe set was designed and later used to determine the mRNA of Cx43 in each sample of cartilage.

Next, the location of gene dysregulation was also important in answering the question of whether or not these genes played a role in OCD. A primary antibody to Cx43 was purchased from Sigma®. Samples of cartilages were then stained with an ABC mouse system utilizing DAB as the chromagen.

Lastly, using the sequence of Cx43, a short and very specific sequence was selected to act as the oligonucleotide probe to be used for *in-situ hybridization*. This technique allows the visualization of the location of the Cx43 mRNA expression.


Clincial samples obtained from Glaser study
RNA Samples were isolated.
Primers constructed on sequence homologies with other species.
PCR cloned segments using initial primers were sequenced for exact sequence of each gene in horses.
Real time quantatitve PCR primers and probes were constructed in using the exact sequence of each gene.
RT-PCR was done using appropriate primers and probes.
Clinical samples obtained from Glaser study
Tissue samples were fixed and mounted on to microscopy slides.
Immuno-reacted with Cx43 primary antibody and localized with DAB.
(Brain was positive control)
*In-situ* hybridization with Cx43 specific oligonucleotides.
(Brain was positive control)

**Cartilage Specimens and RNA Extraction**

12 total horses were used in this study, 8 with OCD, and 4 with normal cartilage, see Appendix A. RNA was extracted from each sample as described in Glaser et al. RNA samples were stored in -80°C to maintain sample integrity. Cartilage samples were embedded in wax, sectioned at 6μm and mounted.

**Primer Design and Conventional PCR of Target Genes**

Predicted sequences of Cx43 and PRG4 were obtained from the National Center for Biotechnology Information website. Homologies between species were used to design forward and reverse primers for the suspected coding sequence of both genes. Due to the unknown nature of PRG4, multiple primers sets were designed to ensure successful cloning of the entire gene or fragments of the gene. Each primer was then checked against the alignment to ensure specificity and appropriate position. Selected primers are shown in Table 1.

| Primer Name | Sequence (5' -> 3') |
|---|---|
| Cx43 PCR Forward Primers: | AGGCAACATGGGTGACTGGAGT |
| Cx43 PCR Reverse Primers: | GATGCTCTCAAGCCTGGATCT |
| PRG4 PCR Forward Primers 1: | CATCTCCACCTCGCAGAATTAC |
| PRG4 PCR Reverse Primers 1: | GGCTATTGAAAGAGCTGCCACT |
| PRG4 PCR Forward Primers 2: | GAGAGGTGGCAGGATTC |
| PRG4 PCR Reverse Primers 2: | GAGCCATGCAATGGGAG |

Table 1. Primers used for conventional PCR of Cx43 and PRG4.

Designed primers were then used in a PCR with cDNA isolated and reverse transcribed from chondrocytes grown in T175 flasks to verify the specificity of the selected primers. To perform the PCR, 50μl reactions were set up, each reaction contained 5μl 10X PCR Buffer, 3μl $MgCl_2$, 1μl 10nM dNTP mix, 37.5μl DEPC

water, 0.5µl Taq polymerase, 1µl of the appropriate primer set (forward and reverse) and 2µl DNA sample (retrieved from chondrocytes). The mixture was transferred to a PCR reaction tube and placed in the RoboCycler Infinity (Stratagene®, La Jolla, CA). The PCR cycling conditions were set at 30 cycles of 30 seconds at 55°C and 1 minute at 72°C. 10µl of the final PCR product was then added to 1µl of Blue Juice Gel Loading Buffer from Invitrogen® in separate 1ml eppendorf tubes. This mixture was then subject to 1% agarose electrophoresis for 30 minutes at approximately 90mV. 1Kb PLUS DNA LADDER (Gibco®, North Andover, MA) was added to each gel to help determine band sizes.

**Cloning PCR-Generated DNA Fragment**

After confirmation that the correct size bands were produced with the appropriate primer sets, the PCR product was purified and ligated into a pre-constructed TOPO vector and transformed into competent *E. coli* cells. A TOPO cloning kit from Invitrogen® was utilized for this purpose. For each TOPO reaction, a master mix was prepared, which included 4µl purified DNA, 1µl salt solution, and 1µl TOPO vector. The mixture was incubated at 37°C for 10 minutes. After incubation, 2µl of this reaction mixture was added to a 10µl of *E. coli* competent cells. This reaction was manually mixed gently and incubated for 5 minutes at room temperature. The cells were then heat shocked for 30 seconds at 42°C in order for the cells to complete the ligation. 250µl of LB broth was added to the cells. The tubes were then capped tightly and placed in a horizontal shaker at 37°C for one hour.

Two LB-ampicillin plates were used for each gene. One plate was covered with 50µl of the bacterial broth, with the other one covered with 200µl of the same

broth in order to obtain different colony densities on each plate. Care was taken to ensure that glass spreaders were soaked in ethanol and heated after every use to assure minimal contamination. The plates were then incubated at 37°C overnight.

Individually isolated colonies were transferred to a patch plate, an ampicillin agarose plate numerically divided to correspond to each selected colony in order to easily isolate and propagate each selected colony for storage and future use. The colonies from the original ampicillin plates were selected and carefully picked using a small pipet tip to transfer bacteria to the corresponding box on the patch plate. One pipet tip was used for each colony. The same pipet tip was then dipped into the corresponding PCR reaction tube for colony PCR. The patch plate was then allowed to grow overnight in the 37°C incubator.

For the colony PCR, master mixes for each gene was prepared using the same protocol as above. The reaction product was then run on a 1% agarose gel electrophoresis to determine size of the DNA product. Colonies with appropriate size products were allowed to grow further to provide sufficient sample for sequencing.

Several appropriate colonies were added to 5ml of LB broth with 10µl of 100mg/ml ampicillin. Centrifuge tubes containing the broth mixture were placed in 37°C shaker overnight. After 24 hours, PureLink Quick Plasmid Miniprep Kit from Invitrogen® was used to isolate all plasmids containing the appropriate gene. Concentration of plasmid per samples was checked with NanoDrop® Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmingotn, DE). The samples were then sent to Cornell University Life Science Core Laboratories Center for sequencing of each plasmid. Results of each sequenced plasmid were re-

constructed in MegaAlign (DNASTAR, Madison, WI). Multiple samples of the same sequence were aligned and compared to ensure consensus between each other. It was then compared to known sequences from other species and finally compared to the equine genome.

**Quantitative Real-Time PCR**

Based on the sequences analyzed, two oligonucleotide primer-probe pairs were selected using PrimerExpress 2.0 (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers and probes are shown in Table 2.

For the real-time RT-PCR, the purified RNA samples were analyzed using TaqMan® One-Step RT-PCR (ABI Prism 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA) and associated SDS 2.1 software to quantify gene expression. In addition to Cx43, and PRG4, 18S rRNA was used as a control gene.

| Primer Name | Sequence (5' -> 3') |
|---|---|
| Cx43 Taq Forward Primer: | TGCCGTCTACACTTGCAAAAGA |
| Cx43 Taq Reverse Primer: | CACCAGCATGAAAATGATGAAGA |
| Cx43 Probe: | CCTGCCCGCATCAGGTGGA |
| PRG4 Taq Forward Primer: | GGAAGCACCGCAGATTCG |
| PRG4 Taq Reverse Primer: | TGGTGTGTGTTTGAGAAGGTCCTA |
| PRG4 Taq Probe: | AGACGCCGCTTCCAACGGGC |

Table 2. Primers and probes used for quantitative real-time PCR.

A seven 10-fold serial dilution plasmid standards was prepared to derive a standard curve, along with a no template control. Standard precautions were taken throughout the PCR process to avoid cross-contamination. RNA samples were diluted

to 5ng/μl to standardize concentrations. Master mix for each gene included: 5μM forward and reverse primers, 0.2 μl of 10μM probe, with 10μl of 2X Universal Master Mix, and 0.5μl of 40X Multiscribe®, the latter two being from TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA). Next, 45μl of the appropriate master mix was added to 5μl of each RNA sample. All samples were vortexed, and 20μl of each sample was added in duplicate wells in a MicroAmp® Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA). The RT-PCR was carried out with the ABI Prism 7900 Sequence Detection System. During amplification, the ABI Prism sequence detector monitors fluorescence emission at every thermal cycle. The threshold cycle represented the cycle at which a significant amount of fluorescence can be detected.

**Statistical Methods**

SDS 2.1 software was used to display the RT-PCR results. This data was then exported to Microsoft Excel® in order to normalize Cx43 quantity means to 18S rRNA quantity means. Statistical analysis of Cx43 expression and its comparisons between normal and OCD lesion in cartilage data was done by using Statistix® 8.0 software (Analytical Software, Tallahassee, FL). Further statistical testing was conducted by ANOVA using the Tukey HSD tests for multiple comparisons with a P-value of less than 0.05. Plot was created using SigmaPlot® 2000 (SPSS Inc, Chicago, IL) Data is presented as means +/- standard deviations.

**Immunohistochemistry of Cx43**

Slides with fixed sections of normal and OCD cartilage were subject to polyclonal mouse Cx43 antibodies (Sigma, St. Louis, Missouri). The chromogen used

was diaminobenzidine (DAB) as part of the mouse ABC Staining System (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Anti-mouse primary antibody was used as a negative control while equine brain was used as the positive control. Staining protocol and outline of case identification numbers can be found in the Appendix B.

### *In-situ* Hybridizationof Cx43

Slides with fixed sections of normal and OCD cartilage were subject to *in-situ* hybridization with oligonucleotides specific to Cx43. Oligonucleotide probes were chosen due to its resistance to RNases and their small size. The small size made them ideal for easy penetration into the tissue. Since the specific sequence of Cx43 was now known, a very specific probe was made. Three anti-sense oligonucleotides were synthesized to bind to Cx43 mRNA, while three sense oligonucleotides were synthesized to act as negative controls. All six oligonucleotides were checked to be all exclusive to the Cx43 equine gene. The sequences were put into BLAST, with the only matching result being the Cx43 gene. The oligos ranged from 46 to 49 bp. The commercially synthesized oligonucleotides were tagged with Ulysis Alexa Fluor® 488 Nucleic Acid Labeling Kit (Molecular Probes, Eugene, OR). Hybridization protocol and an outline of case identification numbers can be found in Appendix C. RNase was used on negative controls and the equine brain was used again as positive control.

| Oligonucleotide Name | Sequence (5'->3') |
|---|---|
| Probe 69-117 (anti-sense) | TGTCCCCAGTAGCAGGATTCGGAAAATGAAAAGGACTGACACACACC |
| Probe 340-385 (anti-sense) | GCTTCAAGTGCATCTCCACATTGACACCATCAGTTTGGGCAACTTT |
| Probe 421-469 (anti-sense) | TGATGTAGGTTCGCAGCAAGCCCCCTCGCATTTTCACCTTGCCATGCTC |
| Sense probe 1 | GAGCATGGCAAGGTGAAAATGCGAGGGGGCTTGCTGCGAACCTACATCA |
| Sense probe 2 | GGTGTGGCTGTCAGTCCTTTTCATTTTCCGAATCCTGCTACTGGGGACA |
| Sense probe 3 | AAAGTTGCCCAAACTGATGGTGTCAATGTGGAGATGCACTTGAAGC |

Table 3. Cx 43 anti-sense and sense oligonucleotides used for the *in-situ* hybridization study.

# Results

## Identification of Cx43

The gel electrophoresis of the conventional PCR with the proposed primers and chondrocyte cDNA showed a band at the expected 1200 bp mark (Figure 4, lane 2). This result confirmed that the primers designed and synthesized based on sequences from other species were accurate and were able to amplify a clone to be used for sequencing of the Cx43 gene in the horse.

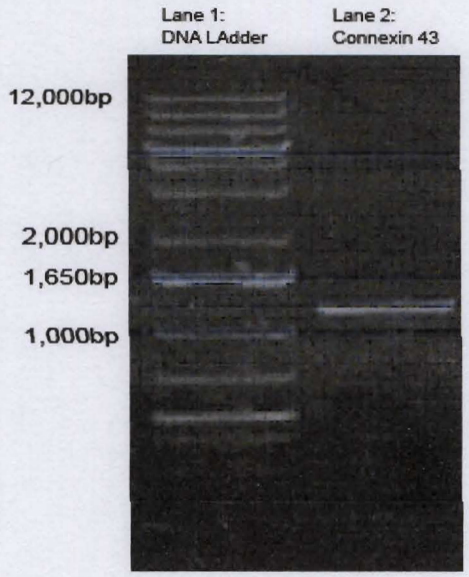



**Figure 4. Agarose gel of Cx43 PCR. Lane 1 shows the DNA ladder, Lane 2 shows the result of the PCR with new primers.**

## Sequence Analysis

After cloning the PCR product, the purified plasmid DNA was sent to be sequenced at Cornell Biotechnology Core Laboratories. The sequences were aligned using MegaAlign (DNASTAR Inc, Madison, WI). The sequence was then entered into equine genome BLAST for confirmation. The 1149 bp sequence returned from BLAST with 99.9% confirmation, the only difference at nucleotide 1122 of this sequence. Multiple samples sequenced obtained from Cornell University Core Laboratory supported the below sequence. The equine sequence of the Cx43 is as follow:

5'- ATGGGTGACTGGAGTGCCTTAGGCAAACTCCTTGACAAAGTTCAAGCCTATTCCACAGCCGG

AGGGAAGGTGTGGCTGTCAGTCCTTTTCATTTTCCGAATCCTGCTACTGGGGACAGCGGTTGAG

TCAGCCTGGGGTGACGAGCAGTCTGCCTTTCGTTGTAACACTCAGCAACCTGGTTGTGAGAACG

TCTGCTATGACAAGTCCTTTCCAATCTCTCACGTGCGCTTCTGGGTCCTGCAGATCATATTTGTGT

CTGTCCCCACGCTCTTGTACCTGGCTCATGTGTTCTATGTGACGCGAAAGGAAGAGAAACTGAA

CAAGAAAGAGGAGGAACTCAAAGTTGCCCAAACTGATGGTGTCAATGTGGAGATGCACTTGAA

GCAGATTGAAATCAAGAAGTTCAAGTACGGCATTGAAGAGCATGGCAAGGTGAAAATGCGAG

GGGGCTTGCTGCGAACCTACATCATCAGTATCCTCTTCAAGTCGCTCTTTGAGGTGGCCTTCTTG

CTGATCCAATGGTACATCTATGGATTCAGCTTGAGTGCCGTCTACACTTGCAAAAGAGATCCCTG

CCCGCATCAGGTGGACTGCTTCCTCTCGCGTCCCACAGAGAAAACCATCTTCATCATTTTCATGC

TGGTGGTGTCCTTGGTGTCTCTTGCCTTGAACGTCATCGAACTCTTCTATGTCTTCTTCAAGGGT

GTTAAGGATCGTGTGAAGGGAAAAGGTCATCCTTACCACACCACCATCGGCCCGCTGAGCCCCT

CCAAAGACGTTGGATCTCCAAAATACGCTTATTTCAATGGCTGCTCTTCACCAACCGCTCCTCTCT

CGCCCATGTCTCCTCCCGGGTACAAGCTGGTTACCGGAGACAGAAACAATTCTTCCTGCCGCAA

TTACAACAAACAAGCAAGTGAGCAAAACTGGGCTAATTACAGTGCAGAACAAAATCGAATGGG

GCAGGCAGGAAGCACCATCTCTAACTCCCACGCACAGCCTTTTGATTTCCCTGATGACAACCAG

AATCCTAAAAAACTGGATACTAGGCACGAACTGCAACCGCTAGCCATTGTGGACCAGGGGCCTT

CGAGCAGAGCCAGCAGTCGCGCCAGCAGCCGGCCCCGGCCTGATGACCTGGAAATCTAG-3'

**Identification of PRG4**

Using the same strategy as Cx43, we successfully obtained the two DNA fragments with commercially synthesized primer sets. The size measured by running a conventional PCR showed a predicted band at about the 600 bp mark by using primer set 1 (Figure 5, Lane 4) and a predicted band at the 200 bp mark by using primer set 2 (Figure 5, Lane 2). This result confirmed that the primers synthesized

were able to clone portions of the equine PRG4 gene using sequence homologies between the difference species.

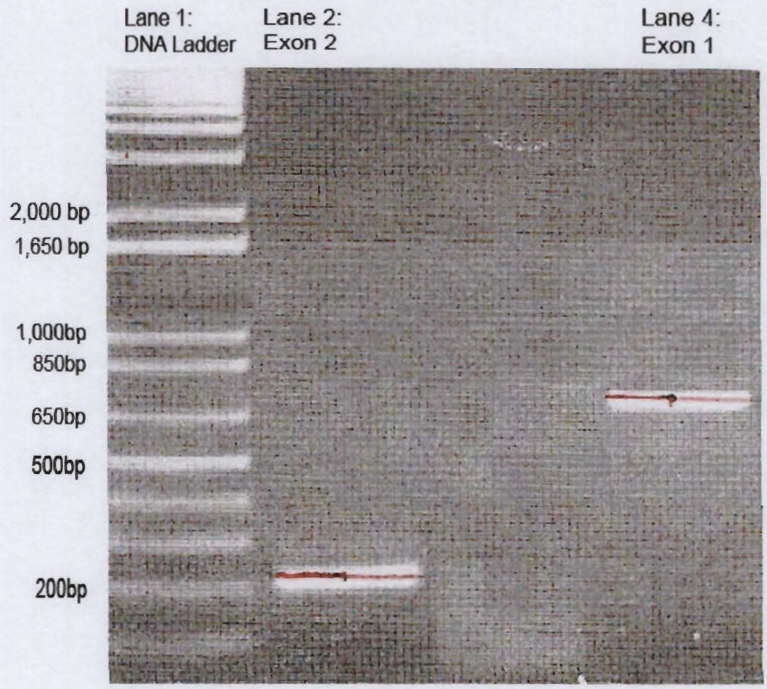



**Figure 5. Gel electrophoresis of PRG4 conventional PCR. Lane 1 shows the DNA band markers; lane 2 shows the result of using primers set 2 to isolate exon 2; lane 4 shows the result of using primer set 1 for primers for exon 1.**

**Sequence Analysis**

From previous sequences, predicted sequences, and the literature, PRG4 was suspected of having many exons. Due to the many exons in the PRG4 gene, only two were sequenced. This allowed adequate sequence to design a specific probe. Upon receiving the sequence from Cornell Biotechnology Core Laboratories, the sequences were aligned using MegaAlign (DNASTAR, Madison, WI). The sequence was then entered into equine genome BLAST for confirmation, however there was no matching sequence in the horse genome. When sequences were put into BLAST of other species, such as human, cow, or mouse, the sequence returned with over 80%. This fact shows that the sequence that follows does seem likely to

be the PRG4 gene in the horse, however, at the current moment it is not yet annotated in the published genome. The equine sequences of PRG4 are as follow:

Exon 1:

5'-AGGTGGCAGGATTCAGCAGTATATTTATAAACAGGAACCCATCAGAAAGTGCCCG
GGAAGGCGGCCTGCTATCCATTATTCGGTGTATGGGGAAGCACCGCAGATTCGGAGA
CGCCGCTTCCAACGGGCTATAGGACCTTCTCAAACACACACCATCCGAATTCACTATTC
ACCCGTCAGAGTCTCTTATCAAGACAAAGTGCCCTCCACAGATTTCCTCCATAATGAAG
TTAAAGTGAGTACGCTCTGGAGAGGACTTCCCGATACGGTTACTTCCGCTATATCACTG
CCCAACCTCAGAAAACCCGATGGCTACGATTACTATGCCTTTTCTAAGGATCAATACTA
TAACATCGATGTGCCTAGTAGAACAGCAAGAGCAATTACTACTCGCTCTGGGCAGACC
TTATCCAAAGTCTGGTACAACTGTCCTTAGACCGATGGGCAAAGGAAGAGTCGACTAA
TTAAAAGGAAGAAAAGAATGACACTGAAATACATTTTATTAATAAAGAATGTTGAGAT
AAGCATACCAATTTAAATACAAAAATGTTTTTAAACTCGACAATCATTACACTAAAACA
GATGTGACAATCTTATTCACATTTATTACAGTTTGTAGACATTTAATTAATGTTTCCTCT
ATTTATTCCTCCTCTCCCTCCCATTGCATGGCTCA-3'

Exon 2:

5'-ATTCGCCCTTCATCTCCACCTCGCAGAATTACTGAAGTTTGGGGTATTCCCTCCCCCA
TTGATACTGTTTTCACCAGATGCAACTGCGAAGGAAAAACTTTCTTCTTCAAGGATTCT
CAATACTGGCGTTTCACCAATGATATAAAAGATGCAGGGTATCCCAAACTAATTTCAAA
AGGATTTGGAGGACTAAGTGGAAAAATAGTGGCAGCTCTTTCAATAGCCA-3'

**Cx43 Expression in Normal and OCD Cartilage**

To determine the expression level of Cx43, quantitative real-time PCR was performed to measure Cx43 mRNA levels in normal cartilage and OCD cartilage. The expression of Cx43 in normal cartilage average 31.35 copies/ng of RNA with

standard deviation of 8.24 copies/ng of RNA. The expression of Cx43 in OCD affected cartilage had a significantly higher level with an average of 216.52 copies/ng of RNA along with a standard deviation of 187.63 copies/ng of RNA. The expression of Cx43 in OCD cartilage was over 7-folds higher than its expression in normal cartilage ($P<0.05$) (Figure 6).

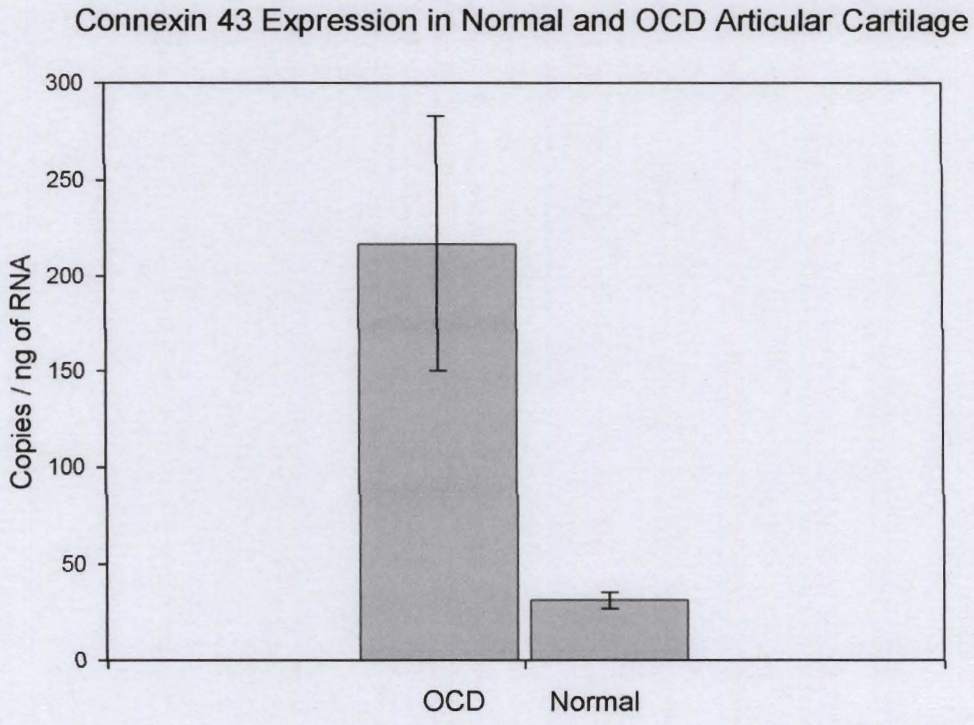



**Figure 6. RT-PCR of Cx43 in OCD and Normal cartilage. OCD cartilage shows an average of 216.52 copies/ng of RNA, significantly higher than the normal cartilage expression of 31.35 copies/ng of RNA; a seven- fold increase in expression in OCD cartilage. N=8 OCD; 4 normal.**

**PRG4 Expression in Normal and OCD Cartilage**

The expression of PRG4 in normal cartilage averaged 66.56 copies/ng of RNA with a standard deviation of 42.74 copies/ng. The expression of PRG4 in OCD affected cartilage averaged 22 copies/ng of RNA with a standard deviation of 15.42 copies/ng. The expression of PRG4 in normal cartilage was over 3 fold higher than the expression in OCD cartilage ($p<0.05$) (Figure 7).

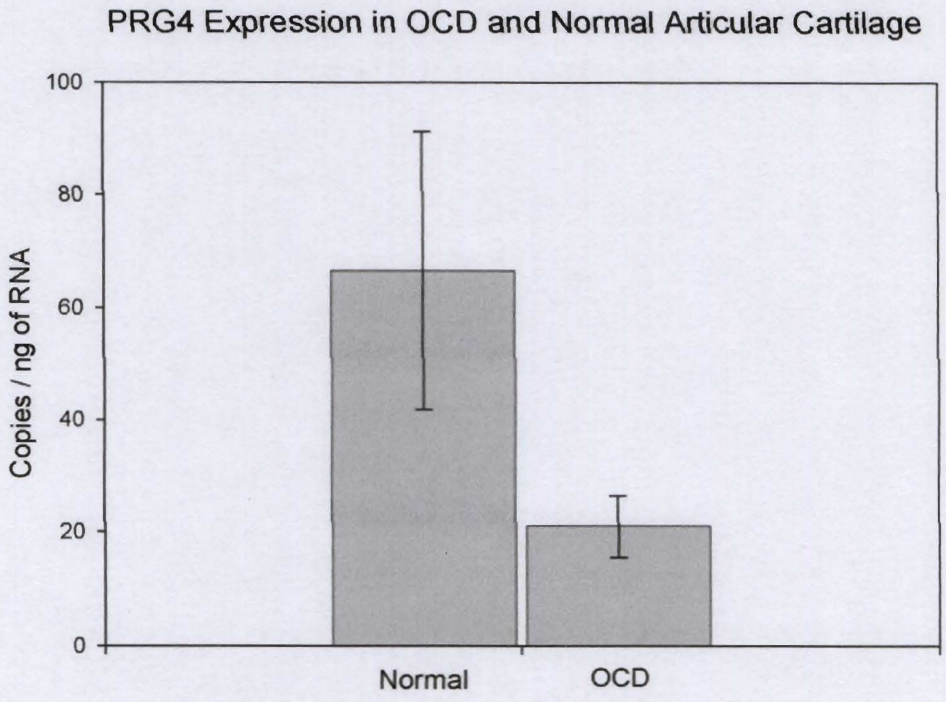



**Figure 7. RT-PCR of PRG4 in Normal and OCD cartilage. Normal cartilage expressed an average of 66.56 copies/ng of RNA, significantly higher than the expression in OCD cartilage of 20.91 copies/ng of RNA. A three-fold increase in expression in normal cartilage. N=8 OCD; 4 OCD.**

**Immunohistochemistry of Cx43 in Normal Cartilage**

In normal samples, Horse DP and Horse 652 (Figures 7A-D) exhibited normal structure of the cartilage and bone junction. When immunoreacted with Cx43 polyclonal antibody (Sigma, St. Louis, Missouri), very low level to no DAB chromogen was evident; a result of low immunoreaction between the antibody and the antigen, Cx43. What little DAB seen was due to the slight edge effect on the border of the tissue. At higher powers of magnification, it was very clear that no DAB chromogen could be seen within the tissue. Figure 7-A illustrates the lack of proliferation of cells or cell clusters along the bony junction. There is also no separation between the cartilage the subchondral bone. Figures 7B-D show the chondrocytes in the cartilage line up in organized columns from the bony junction all the way to the articular cartilage. Figures 7A-D show that normal cartilage does not show evidence of a lesion on the surface of the articular cartilage or the development of a cleft. No DAB chromogen could be seen within the tissue, especially at higher powers of magnification.

## Normal Cartilage Cx 43 Immunohistochemistry

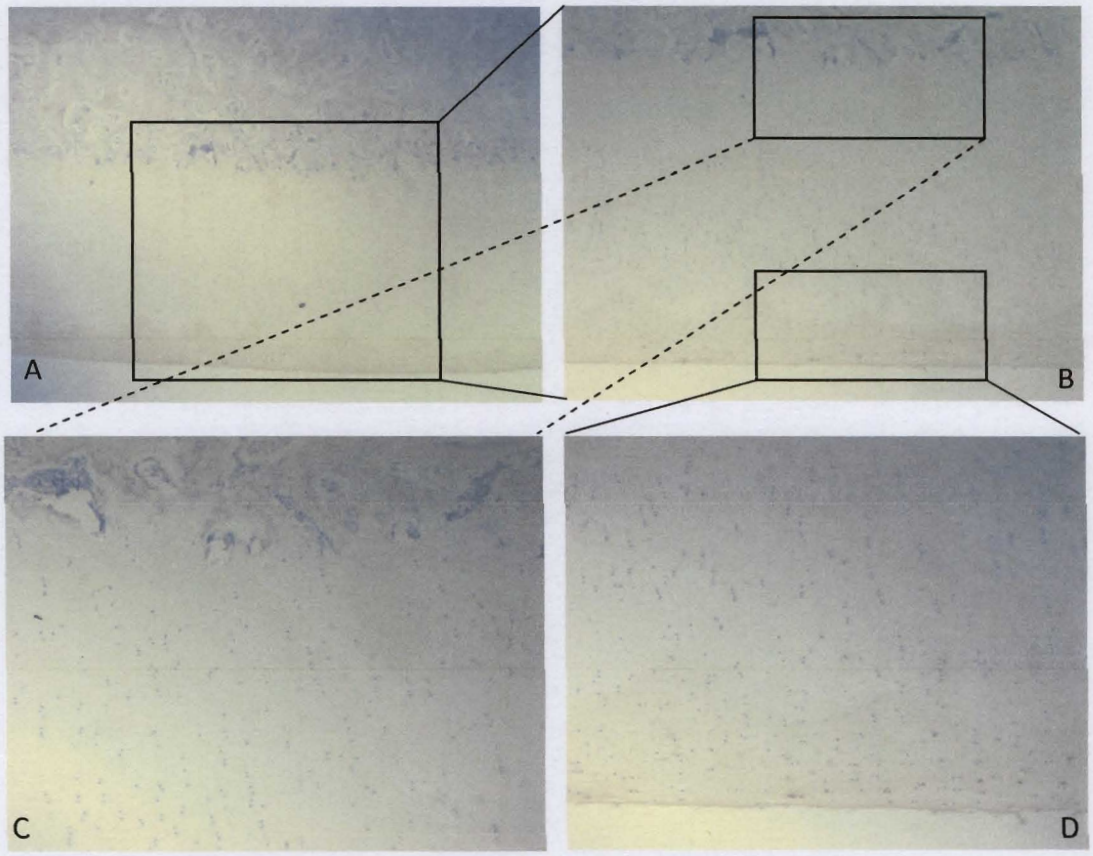


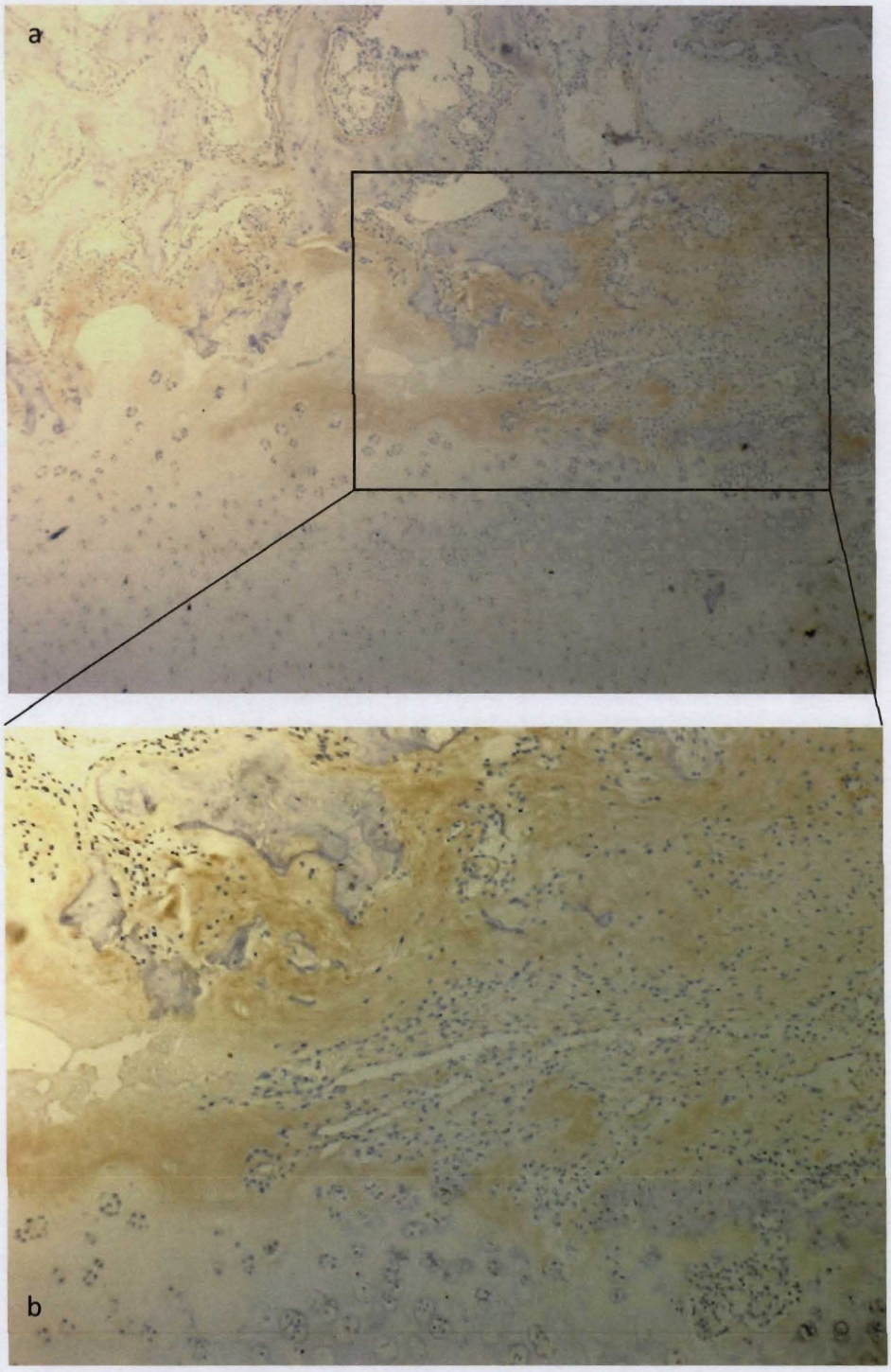

**Figure 7. Normal cartilage samples stained with Cx43 primary antibody according to the protocol located in Appendix B. Figure 7-A is a 25X of DP, showing the subchondral bone and cartilage junction all the way to the articular cartilage. Section enclosed by box is enlarged to 7-B. Figure 7-B shows a 50X view of DP, which shows no excess proliferation of fibroblasts and more columnar organized chondrocytes leading all the way to the articular cartilage. Figure 7-C and D shows the 100X view of respective boxes in figure 7-B. Slight visualization of DAB in figure 7-D was due to edge effect, at higher powers of magnification, no chromogen could be see within the tissue.**

## Immunohistochemistry of Cx43 in OCD Cartilage

In OCD cases (Figures 8 a-d), there was an elevated level of immunoreactions of Cx43 in areas of cellular proliferation around the cartilage and bone junction. The clustering of chondrocytes can also be seen distal to the bony junction. In Figure 8-a, the proliferation of cell and the disorganization of the bony junction can be seen with a portion enlarged to figure 8-b. The lower portion of figure 8-b also shows the clustering of chondrocytes that is often found

in OCD. Upon closer examination, along the upper edge of the figure, there was a large amount of red blood cells located in the bony matrix most likely due to the necrosis and bleeding under the cartilage. Figure 8-c more readily shows the elevated immunoreactions as evident by the increased accumulation of DAB around the fibrocartilage and between the chondroclasts and the OCD cleft. The proliferation of cells in figures 8-b,c show that the new cells are more indicative of fibrocartilage than the normal cartilage and chondrocytes that were present beforehand. The distinct transition can be clearly seen going from right to left in figure 8-c. There is also an indication of small separations between the bone matrix and the cartilage. We believe that this will eventually form the cleft that allows the cartilage lesion to separate from the subchondral bone. This may also explain why many lesions are only discovered after trauma to the joint. The trauma provides the force necessary for the small separations to link up with one another and form the eventual cleft. In Figures 8-c, the heavier staining of Cx43 is mostly located proximally to the bony junction. As Figures 8-d goes on to show that the location of staining overlaps with the location of the cells, indicating that the protein expressed is from the newly formed cells in the region. Figure 8-d also shows that the pattern of staining is consistent with the fibrous cells and the surrounding structure.

**OCD Cartilage Cx 43 Immunohistochemistry**



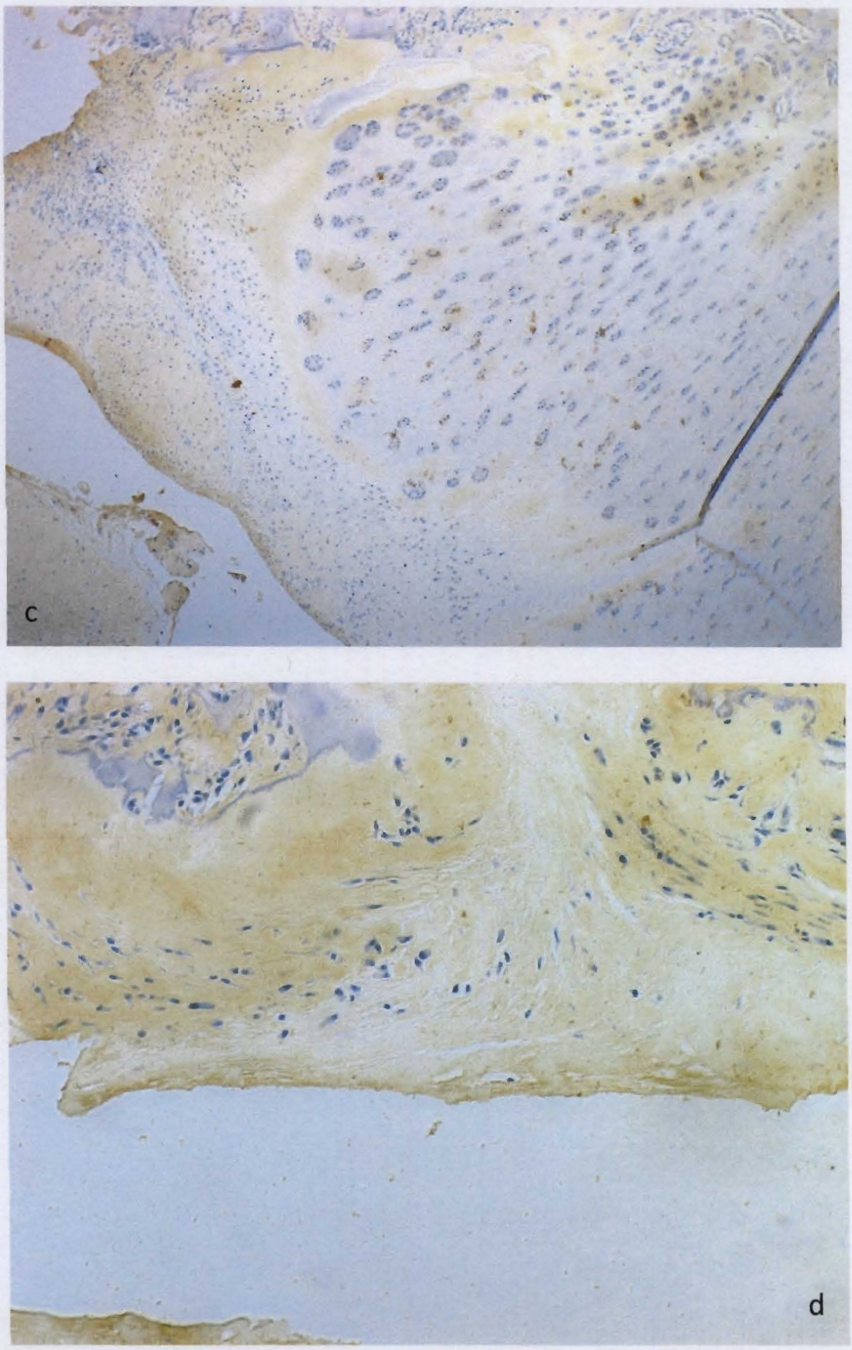



**Figure 8. OCD cartilage samples stained with Cx43 primary antibody according to protocol located in Appendix B. Figures 8-a and 8-b show the 25X and 50X view of 867467L respectively. They demonstrate that the heavier staining by DAB is located around the area of fibroblast proliferation that disrupts the normal subchondral bone cartilage junction, which leads small gaps that may lead to larger clefts and chondrocytes clusters proximal to the area of Cx43 up-regulation. Figure 8-c shows a 100X view of case 867467L. Note the heavier staining of DAB above the chondrocytes clusters, also note the transition from relatively normal cartilage to more fibrocartilage from right to left. The higher staining is primarily within the fibrocartilage and between the chondroclasts and the cleft. Figure 8-d is a 200X view of 867467L, which shows that the heavier staining of DAB overlaps with the formation of the cells, and not due to edge effect.**

### *In-situ* hybridization of Cx43 in Normal Cartilage

Normal cartilage was hybridized by the probes as mentioned in section 2.3.1 and according to the protocol located in the Appendix C. As mentioned in section 3.5.2, the significant red blood cells found in the tissue samples created significant amount of auto-fluorescence; this fact hindered the capture of images of the bone matrix, and the capture of images at higher magnifications. However, only very faint fluorescence can be seen in the cartilage distally to the bone cartilage junction as shown in Figure 9-A; continuing to move distally towards the articular cartilage, there is no evidence of fluorescence.

**Normal Cartilage Cx 43 *in-situ* Hybridization**

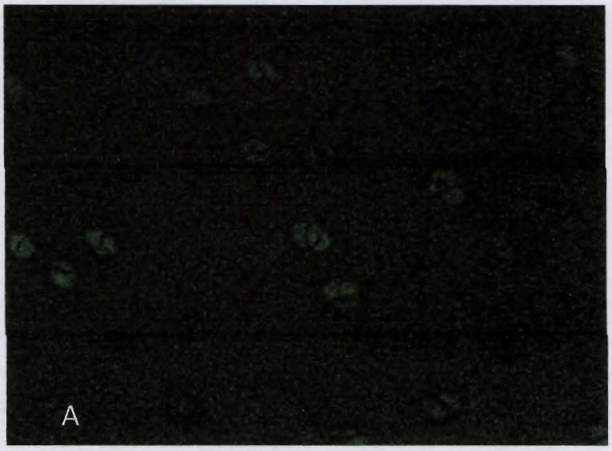



**Figure 9. *In-situ* hybridization of 652 LTR with Cx43 oligonucleotides at 20X magnification. There is very low fluorescence in normal cartilage samples due to the low expression of Cx43 RNA.**

### *In-situ* hybridization of Cx43 in OCD Cartilage

In OCD cartilage, there was significant fluorescence from all areas of the tissue, especially along the bony junction and around the sites of subchondral necrosis and within the chondrocyte clusters, as shown in figure 10. Figures 10-a, b show the fluorescence of Cx43 extensively along the bony junction. This area will eventually become the site of separation and lead to the development of a cleft. In figures 10-e, f, significant fluorescence can be seen along the location of future separation between the cartilage and bone, as hinted by the white dashed line. The location of the fluorescence directly overlaps with the elevated staining seen in immunohistochemistry. In figures 10-a, b, c large clusters of chondrocytes can also be seen with high levels of fluorescence indicating the high levels of expression of Cx43.

## OCD Cartilage Cx43 *in-situ* Hybridization

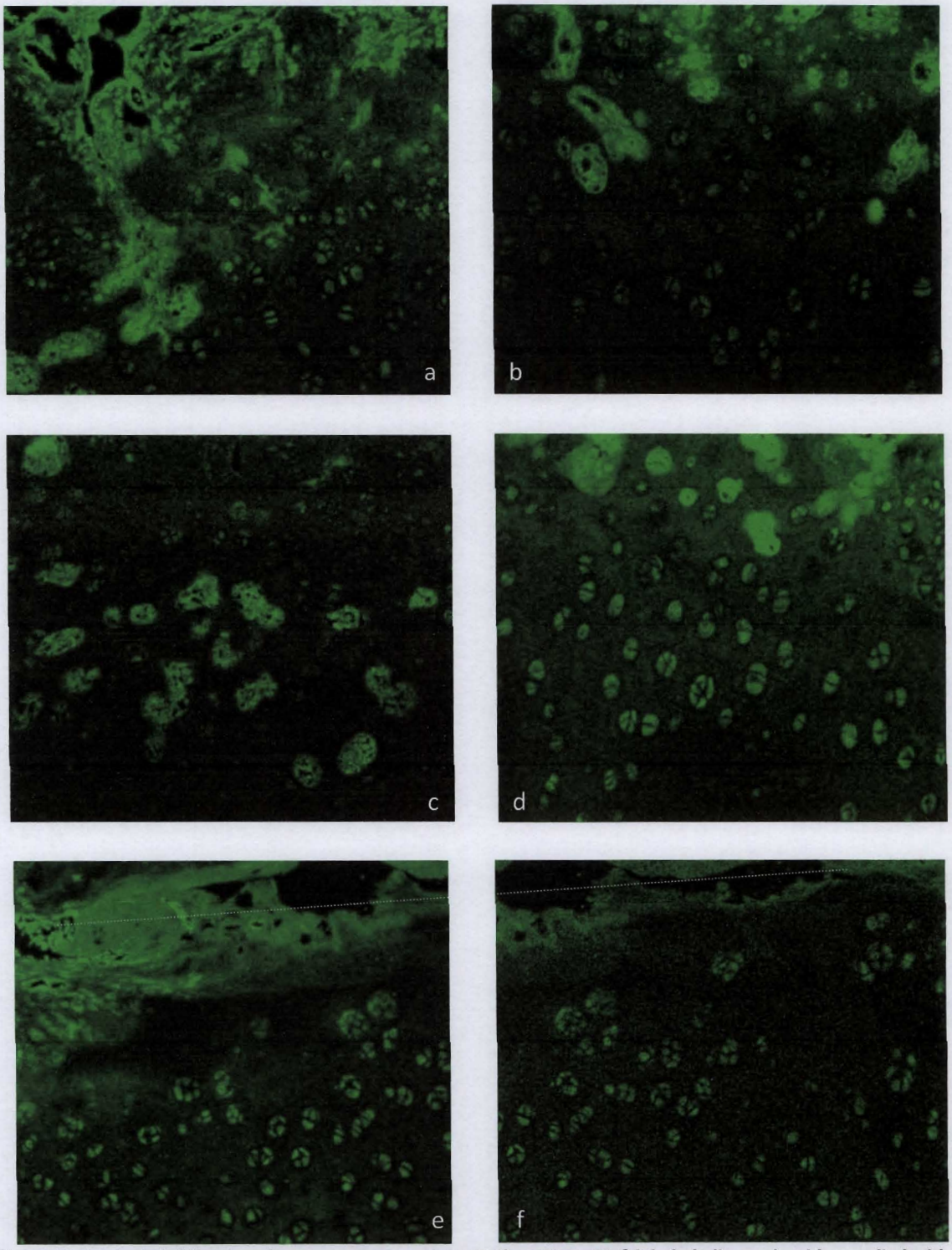



**Figure 10.** ***In-situ*** **hybridization of Horse 867467L with Cx43 Alexa Fluor 488® labeled oligonucleotides applied with protocol located in Appendix C at 400X. There is much greater fluorescence along this junction than normal cartilage located in Figure 9-A. Figures 10-d,e,f all prominently show the prolific osteoclasts/chondroclasts expressing high levels of Cx43 in the base of OCD. Both 10-e and f show the location of the cleft, which overlaps with the higher expression of Cx43 gene.**

**Challenges and Future Direction**

The main challenge to the quest of understanding the etiology and pathogenesis of OCD has been the fact that clinical samples described in the literature have been caught too late in its process to obtain an accurate picture of the initial stages. Although many of the samples in this study showed early clefting, even this may be too late in the disease process to characterize early events up stream of Cx43 accumulation. Another challenge has been the lack of information describing the role of Cx43 and its role in cartilage. Horses are only taken to the veterinarian after the horse displays discomfort, at which time the OCD is often already too severe to help us understand the early stages of OCD.

One way to solve this problem would be to do a costly routine screening of research horses with the hope that early OCD can be recognized. Even with this method, OCD is only isolated after the disease has progressed enough for visualization with diagnostic imaging techniques. Another method would be to develop a knock-in or knock-out genetic models or to use RNA silencing to produce a tissue culture method that replicates the process of OCD.

New and exciting technology will also help our understanding of OCD. A new technique of laser capture microdissector can help us better locate the exact location of gene dysregulation. Currently we are able to see that OCD samples have higher expression of Cx43, but techniques like laser capture can tell us if the higher expression of Cx43 is in the articular cartilage or in the subchondral bone matrix.

# Discussion

This study was able to explore the dysregulation of Cx43 and PRG4 in OCD affected cartilage. To our knowledge, it was the first time that Cx43 was cloned from equine tissue, which allowed us construct a very specific DNA probe for later stages. Its expression was also evaluated by quantitative RT-PCR, immunohistochemistry, and *in-situ* hybridization. We have shown that Cx43 is expressed over 7-fold more in OCD cartilage than in normal cartilage, which is fully consistent with the data obtained previously by Glaser et al. But by using RT-PCR, we were able to focus more specifically on the expression Cx43, rather than using the gene-chip method to screen for dysregulated genes. From immunohistochemistry and *in-situ* hybridization this dysregulation is mainly localized in the bone and cartilage junction. As we try to better understand the role of Cx43 in cartilage we look to its role in other tissues.

As Cx43 is better studied in the heart, it seems to play a role in intracellular communication in addition to cardiogenesis. In intracellular communication, Cx43 is involved with the influx propagation in the ventricles, and the inactivation of Cx43 in the cardiomyocytes predisposes the animal to the development of cardiac abnormalities.[22] In another study, skeletal myoblasts (SMs) that did not express Cx43 did not protect against ventricular tachycardia (VT) in mice, whereas SMs genetically engineered to express Cx43 showed the same protection against VT as that of embryonic cardiomyocytes (eCMs). The eCMs transplanted were found to improve the electrical coupling between surrounding cells by increasing the conduction velocity, which was dependent upon the expression of Cx43.[28] Another study found similar results of slowed ventricular activation if Cx43 was completely lost during development.[18] Although it is difficult to think electrical conduction is

taking place in the subchondral junction, it is entirely possible for the Cx43 to be transporting calcium ions and similar ions as osteoclasts and osteoblasts are trying to remodel the subchondral bone and cartilage junction. This seems to be in agreement with the discovery that gap junctions allow for the rapid cellular communication between cells which is possible even in the articular cartilage.[2]

Cx43 is also one of the most abundant connexins in the brain and is highly expressed during development.[30] Similarly in astrocytes, Cx43 is again involved in the transmission of low-molecular weight materials, such as glucose and its metabolites, from blood vessels to distal neurons.[14,29] In a different study examining the mechanism to which axon trajectory can be altered in response to remote neuronal activity, it was found that the underling guidance mechanism involves astrocyte $Ca^{++}$ waves, which a Cx43 antagonist carbenoxolone has the ability to abolish. [29] Once again the Cx43 is tied back to the ability to transfer calcium ions. Inflammatory response in connection with Cx43 was determined in a study investigating optic nerve ischaemia, in which oxygen-glucose deprivation induced an up-regulation of Cx43, yet when an application of Cx43 antisense oligodeoxynucleoties was added to the post-ischaemic nerve segment, Cx43 up-regulation was significantly reduced, reducing the subsequent spread of injury and the resultant inflammatory response as well.[5] In a similar study, Cx43 was highly up-regulated after spinal cord injury, but with the addition of mimetic peptides, Cx43 and the number of glial fibrillary acidic protein positive astrocytes were reduced, showing that the regulation of Cx43 hemichannel openings using mimetic peptides is a useful treatment for reducing the spread of damage after a spinal cord injury.

From these studies, Cx43 plays an important role in the inflammatory process of ischemia and spinal cord injuries. It is entirely possible that Cx43 plays a similar role in cartilage, by transmitting cytokines that prolong the inflammatory process which incurs more damage to the surrounding tissue. This supports our initial hypothesis that Cx43 in cartilage plays an important role in prolonging the inflammatory process.

Recently, Mori et al. conducted a study that examined the role Cx43 in the process of wound healing, in which they showed that down-regulation of Cx43 appeared to improve the rate and quality of healing. With the knockdown of Cx43, there was a significantly faster healing process, associated with the increase in TGF-β1, collagen type I, and general content at the wound site, as well as the rate of fibroblast migration into the wound to lay down collagen matrix. This suggests that the knockdown of Cx43 may be beneficial in subchondral bone repair.

Taken together, Cx43 acts to prolong the inflammatory process by transmitting inflammatory cytokines and chemokines. The knockdown or disruption of Cx43 would offer potential new therapies for controlling the inflammatory process after the onset of OCD, and possibly even hinder the early development of OCD. Our understanding of this disease and its connection with Cx43 can only be improved as more studies demonstrate the role of Cx43 in cartilage. With our current understanding, it appears that Cx43 is a secondary molecule that proliferates in fibroplasias and is not a primary molecule that would explain the etiology of OCD. From the little work that has been done in the orthopedic field, it was found that Cx43 plays a critical role in regulating the ability for ostoblasts to respond to fibroblast

growth factor 2 (FGF2).[21] Since FGF2 is an important part in angiogenesis, future research should look at the presence of other molecular markers for angiogenesis in OCD.

The location of higher immune-reactions was partially expected since the subchondral bone junction is where OCD first develops and where most structural changes occur. However, the over-expression of Cx43 was not between neighboring chondrocytes as other studies have demonstrated; it was along the cleft and surrounding the osteo/chondroclasts. The fluorescence seen in *in-situ* hybridization was where we had expected, mainly being expressed in the osteo/chondroclasts. This fact may offer an explanation for the location of higher Cx43 expression. From its site of translation in the osteo/chondroclasts, Cx43 must move towards the site of the greatest need or the site of the greatest structural damage, which is along the cleft of an OCD lesion.

The dysregulation of PRG4 was also further characterized in this study. In OCD affected cartilage, the production of PRG4 was three times lower than that of normal cartilage. In several studies, pro-inflammatory cytokines IL-1β and TNF-α have been found to be responsible for marked reduction in PRG4 protein levels.[7,17,31] At the same time, it was found that PRG4 mRNA expression is stimulated by TGF-β1 and BMP.[20,35] TGF-β seems to be the more critical regulator of PRG4 since BMP did not elicit as great of a magnitude in PRG4 stimulation.[25] However, another role of PRG4 may actually inhibit the repair. It was found that PRG4, which normally contributes to cartilage lubrication, can inhibit integrative cartilage repair. This has

the desirable effect of preventing fusion of opposing surfaces of articular cartilage, but has the undesirable effect of inhibiting integrative repair.[8]

The down-regulation of PRG4 is inconsistent with the results from Glaser et al, in which growth factors that contribute to the anabolic stimulus such as TGF-β1and BMP ranked 9th and 16th in abundance, indicative of self repair.[11] We believe that this inconsistency is due to the fact that IL-1β and TNF-α are also highly expressed in the cartilage which is able to cancel the effects of TGF-β1 and BMP. It has been shown in induced experimental arthritis IL-1α and IL-1β is expressed 125-fold higher and TNF-α is expressed 200-300 fold higher in the synovial fluid.[1] Thus, it is entirely possible for the inflammatory cytokines to overpower the anabolic stimulus provided by the higher expressed TGF-β1and BMP.

All these studies show that cartilage secretion of PRG4 is highly regulated by certain cytokines and growth factors, which goes to show that the biochemical environment is an important regulator of PRG4 content during cartilage injury or repair. With this in mind, we believe that PRG4 is a just a spectator gene that is caught in the molecular changes and not a major player in the etiology or pathogenesis of OCD.

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# Appendix

# Appendix A

| | label on tube | Site | OCD/ normal | entire ID: hospital # or name |
|---|---|---|---|---|
| 1 | **467 L.LTR** | Left stifle LTR | OCD | 867467 |
| 2 | **467 R.LTR** | Right stifle LTR | OCD | 867467 |
| 3 | **508 L.LTR** | Left stifle LTR | OCD | 854508 |
| 4 | **508 R.LTR** | Right stifle LTR | OCD | 854508 |
| 5 | **858 L.LTR** | Left stifle LTR | OCD | 869858 |
| 6 | **858 R.LTR** | Right stifle LTR | OCD | 869858 |
| 7 | **769 R.LTR** | Right stifle LTR | OCD | 869769 |
| 8 | **769 L.LTR** | Left stifle LTR | OCD | 869769 |
| 9 | **Fi LTR** | Stifle LTR | normal | Finningen |
| 10 | **DP LTR** | Stifle LTR | normal | DP |
| 11 | **652 LTR** | Right Stifle LTR | normal | 869652 |
| 12 | **BeeDee LTR** | Stifle LTR | normal | BeeDee |

Appendix A. Descriptions of cases used for this study. 8 OCD cases and 4 normal cases.

## Appendix B

### IMMUMOHISTOCHEMISTRY – CONNEXIN 43

Date written: 2/8/08
Updated: 8/7/08

**Sections:**

I: Equipment
II: Supplies and Reagents
III: Reagent Preparation
IV: Experimental Protocol

**Author:** Yang Gu

**EQUIPMENT**

Fume Hood
Viral Incubator

**SUPPLIES AND REAGENTS**

ABC Staining System (sc-2018) (C3-126 refrigerator)
Acid Alcohol (See Reagent Preparation) (C3-126, cabinets)
Ammonia Water (See Reagent Preparation) (C3-126, cabinets)
Ammonium hydroxide [$NH_4OH$], Fisher A669-12 (cabinet under hood, C3-126)
Biogenex Negative Control (Mouse Serum), HK119-7M (C3-126, refrigerator)
Connexin 43 rabbit anti-equine (C3-176, -20 freezer)
Concentrated HCl, A144-500 (cabinet under hood, C3-176)
Coverslips, Fisher Finest 24x40, #12-548-55 (C3-126, drawers near hood)
Eppendorf pipettor and yellow micropipette tips (C3-126, bench)
100% Ethanol, Pharmca UN1170, 200 Proof (C3-126, viral room)
95% Ethanol, Pharmca UN1170, 190 Proof (C3-126, viral room)
Glass Staining Dishes with Glass Lids, 23 of each (C3-126, drawers)
Harris Hematox, Fisher SH30-500D (C3-126, shelves)
Hyaluronidase Type 1-S, Sigma H-3506 (C3-126, freezer)
PAP Pen (C3-126, bench)
0.5% Peroxide/PBS (See Reagent Preparation)
3% Peroxide, Leader® [store-bought] and 3% Peroxide dropper bottle (C3-126, Refrigerator)
Phosphate Buffered Saline (See Reagent Preparation)
Phosphate Buffered Saline/ 0.3% Triton X-100 [See Reagent Preparation] (C3-126, cabinet)
Refrax Mounting Medium, Anatech Ltd. #711 (C3-126, cabinet under hood)
RO Water (Tap water can be used as well)
20mM Sodium Acetate, FW= 82.03 [$CH_3COONa$] (See Reagent Preparation) (C3-126, cabinet)
Three Staining Racks with Metal Holder (C3-126, drawers)
Three Tupperware Containers [Humid Chambers, HC] with Lids & Black Racks (C3-126, cabinets)
Transfer Pipettes (C3-126, cabinets)
Triton X-100, Lot 131617 (C3-178, cabinets)
Tris HCl Buffer [See Reagent Preparation] (C3-126, cabinets)
Trizma® HCl [tris], Sigma T-6666 (C3-178, cabinets)
Xylene, Histological Grade, Fisher Scientific UN1307 (C3-126, Hood)

**REAGENT PREPARATION:**

**The following are prepared in advance:**

**20mM Sodium Acetate**:

- Weigh 0.82 g $CH_3COONa$

- Add to 500 mL RO Water

**Phosphate Buffered Saline [PBS]:**

- Weigh the following:
  - 7.75 g NaCl
  - 1.5 g $K_2HPO_4$
  - 0.2 g $KH_2PO_4$
- Add to 800 mL RO water.
- Adjust pH to 7.6 ± 0.2 {Titrate with 1 N HCl or NaOH as needed}
- QS to 1 Liter

**Tris HCl Buffer**: [used in rinse step and with DAB]

- Weigh the following:
  - 6.1 g Tris [Trizma base; T-1503]
  - 37 mL 1 N HCl -OR- 3.06mL of 12.1 N HCl
- Add to 800 mL RO Water
- Adjust pH to 7.4
- QS to 1 Liter

**PBS/0.3% Triton**: [used to dilute primary antibody]

- Measure the following:
  - 150 mL PBS
  - 450 µLs Triton X-100 Stock
- Mix well in a beaker

**70% Ethanol**:

- Measure and mix the following:
  - 700 mL 100% Ethanol (EtOH)
  - 300 mL RO water

**80% Ethanol**: 250 mL fills glass staining dish

- Measure the following:
  - 200 mL of 100% Ethanol (EtOH)
  - 50 mL of RO water
- Add to glass staining dish

**Acid Alcohol**:

- Measure the following:
  - 10 mL 12N HCl
- Add to 990 mL of 70% EtOH

**Ammonia Water**:

- Measure the following:
  - 40 drops $NH_4OH$
- Add to 1 Liter of RO water.

**The following must be prepared the DAY of experiment**:

***{NOTE: the following based on 36 slides with two medium bone tissue sections/slide}***

**Hyaluronidase**:

- Measure the following:
  - 50 mg Hyaluronidase
- Add to 10 mL Sodium acetate and mix well.

**0.5% $H_2O_2$/PBS**:

- Measure and mix the following:

10 mL PBS
2 mL 3% Peroxide

**Primary Connexin 43 Antibody and Negative Control**:

- Thaw Antibody about 20 min prior to use
- Measure the following:
  3 µL primary antibody
  597 µLs of PBS/.3% Triton X-100
- Mix the above well in a conical tube
- Final dilution should be 1:200 [Antibody/ dilute].

## EXPERIMENTAL PROTOCOL

**Helpful Notes**:

A. Make solutions fresh EACH & EVERY time during this process.
B. Keep solutions [esp. Biogenex products] refrigerated as much as possible.
C. Dispense solutions from an eppendorf tube using the yellow micropipette tips
D. Cover chambers at all times to prevent solutions from evaporating and drying tissues.

1. On the day before the experiment label, set up & fill 250 mL of solution into glass dishes as follows: Deparaffinization: 3 dishes for Xylene
   2 dishes for 100% EtOH
   2 dishes for 95% EtOH
   1 dish for 80% EtOH
   1 dish for RO $H_2O$
   1 dish for PBS

   Counterstain/Dehydrate: 1 dish for Harris Hematox
   1 dish for RO $H_2O$
   1 dish for Acid Alcohol
   1 dish for RO $H_2O$
   1 dish for Ammonia Water
   1 dish for RO $H_2O$
   2 dishes for 95%EtOH
   2 dishes for 100% EtOH
   3 dishes for Xylene
2. Get Humid Chambers warmed up with a blank slide rack in incubator.
   - Add a couple of thoroughly wet paper towels on bottom of chamber.
3. Arrange slides vertically into staining racks.
4. Dip staining rack into Xylene I dish for 5 min.; proceed to then dip rack into Xylene II for 5 min.; then into Xylene III for 5 min.
   - Before proceeding to the next dish, drain off any excess liquid.
5. Dip staining rack into 100% EtOH for 5 min; then dip rack into second 100% EtOH for 5 min.
6. Dip staining rack into 95% EtOH for 5 min.; then dip rack into second 95% EtOH for 5 min.
7. Dip staining rack into 80% EtOH for 5 min.

8. Dip staining rack into RO $H_2O$ for 5 min.

9. Prepare Hyaluronidase during this time period.

10. Dip staining rack into PBS for 5 min.

11. Blot and circle tissue sections with PAP pen; place slides horizontally on humid chamber racks.
    - Do not depress tip of PAP pen, spillage will occur.

12. Dispense 20-50 µL Hyaluronidase onto tissue sections, close chamber lids.
    - Incubate in humid chambers for 60 min. @ 37°C
    - Prepare 0.5% $H_2O_2$/PBS towards end of incubation.

13. Tap off solution on slides and dispense 20-50 µL PBS onto tissue sections.
    - Incubate in humid chambers for 5 min at room temperature.

14. Dispense 20-50 µL 0.5% $H_2O_2$/PBS onto tissue sections.
    - Incubate in humid chambers for 20 min. at room temperature.
    - Prepare primary antibody during this time.

15. Tap off solution on slides and dispense 20-50 µL PBS onto tissue sections.
    - Incubate in humid chambers for 5 min at room temperature.

16. Incubate sections for one hour in 1.5% blocking serum (mixing bottle 1).
    - Incubate in humid chambers for 30 min at room temperature.
    - This step blocks nonspecific protein-binding sites.

17. Tap off protein block (no rinse) and dispense a.) dispense 20-50 µL of primary antibody b.) dispense 20-50 µL **Negative Control** onto other tissue section of one slide.
    - Use extreme care so as to not allow intermixing of these solutions.
    - Incubate in humid chambers for 30 min. at room temperature.

18. Tap off solutions on slide and dispense 20-50 µL PBS onto tissue sections.
    - Incubate in humid chambers for 5 min at room temperature.

19. Dispense 2 drops **biotinylated secondary antibody** (mixing bottle 2) onto tissue sections.
    - Incubate in humid chambers for 30 min. at room temperature.

20. Tap off solution on slide and dispense 20-50 µL PBS onto tissue sections.
    - Incubate in humid chambers for 5 min at room temperature

21. Dispense 2 drops **AB enzyme reagent** (AB mixing bottle) onto tissue sections.
    - Incubate in humid chambers for 30 min. at room temperature

22. Tap off solution on slide and dispense 20-50 µL PBS onto tissue sections.
    - Incubate in humid chambers for 5 min at room temperature.

23. Dispense 2 drops **peroxidase substrate** (substract mixing bottle) onto tissue sections.
    - Incubate in humid chambers for 10 min. at room temperature.

24. Tap off solution on slide and dispense 20-50 µL RO $H_2O$ onto tissue sections.
    - Incubate for 5 min at room temperature.

25. Blot off excess water onto paper towel and arrange slides vertically into staining rack.

26. Dip staining rack into Harris Hematox for 5 min.
    - Filter solution before each use. Solution can be reused.
27. Dip staining rack into RO $H_2O$ for 1 min.
28. Dip staining rack into Acid alcohol for 1-3 quick dips to remove excess stain.
29. Dip staining rack into RO $H_2O$ for 5 min.
30. Dip staining rack into Ammonia water for 2 min.
31. Dip staining rack into RO $H_2O$ for 5 min.
32. Dip staining rack into 95% EtOH for three dips.
33. Dip staining rack into second 95% EtOH for three dips.
34. Dip staining rack into 100% EtOH for 3 min.
35. Dip staining rack into second 100% EtOH for 3 min.
36. Dip staining rack into Xylene I for 3 min.
37. Dip staining rack into Xylene II for 3 min.
38. Dip staining rack into Xylene III for 3 min
39. Blot off excess xylene and begin to coverslip using glass pipe to add 1-3 drops of Refrax mounting medium. Ensure there are no air bubbles.

## Appendix C

### IN SITU HYBRIDIZATION – CONNEXIN 43

Date written: 8/7/08
Updated: 3/23/08

**Sections:**

I: Equipment
II: Supplies and Reagents
III: Reagent Preparation
IV: Experimental Protocol

**Author:** Yang Gu

**EQUIPMENT**

Fume Hood
Viral Incubator

**SUPPLIES AND REAGENTS**

Concentrated HCl, A144-500 (cabinet under hood, C3-176)
Coverslips, Fisher Finest 24x40, #12-548-55 (C3-126, drawers near hood)
Eppendorf pipettor and yellow micropipette tips (C3-126, bench)
100% Ethanol, Pharmca UN1170, 200 Proof (C3-126, viral room)
95% Ethanol, Pharmca UN1170, 190 Proof (C3-126, viral room)
Glass Staining Dishes with Glass Lids, 23 of each (C3-126, drawers)
Phosphate Buffered Saline (See Reagent Preparation)
Phosphate Buffered Saline/ 0.3% Triton X-100 [See Reagent Preparation] (C3-126, cabinet)
Refrax Mounting Medium, Anatech Ltd. #711 (C3-126, cabinet under hood)
RO Water (Tap water can be used as well)
Three Tupperware Containers [Humid Chambers, HC] with Lids & Black Racks (C3-126, cabinets)
Transfer Pipettes (C3-126, cabinets)
Triton X-100, Lot 131617 (C3-178, cabinets)
Tris HCl Buffer [See Reagent Preparation] (C3-126, cabinets)
Trizma® HCl [tris], Sigma T-6666 (C3-178, cabinets)
Xylene, Histological Grade, Fisher Scientific UN1307 (C3-126, Hood)

**REAGENT PREPARATION:**

**The following are prepared in advance:**

**20mM Sodium Acetate**:

- Weigh 0.82 g $CH_3COONa$
- Add to 500 mL RO Water

**3M Sodium Acetate:**

- Weigh 12.3 g $CH_3COONa$
- Add to 50mL of RO Water

**4% PFA in 0.1M PBS**

- 8 g in 100ml of H2O
- 100 ml of 0.2M PBS
- pH=7.4

**Phosphate Buffered Saline [PBS]:**

- Weigh the following:
  - 7.75 g NaCl
  - 1.5 g $K_2HPO_4$
  - 0.2 g $KH_2PO_4$
- Add to 800 mL RO water.
- Adjust pH to 7.6 ± 0.2 {Titrate with 1 N HCl or NaOH as needed}
- QS to 1 Liter

**70% Ethanol**:

- Measure and mix the following:
  - 700 mL 100% Ethanol (EtOH)
  - 300 mL RO water

**50% Ethanol**:

- Measure the following:
  - 100 mL of 100% Ethanol (EtOH)
  - 100 mL of RO water

**Pre-Hybridization Buffer (prepare in 50ml tube, store @-20°C)**

- 4 ml 20X SSC
- 4 g Dextran sulphate
- 10 ml Formamide (deionized)
  - Sonicate for 3-4 hours
- 0.5 ml PolyA (10mg/ml)
- 0.5 ml ssDNA (10mg/ml)
- 0.5 ml tRNA (10mg/ml)
- 2 ml DTT (1M solution)
- 0.2 ml 50X Denhardts

**Hybridization Buffer**

- 0.04 µg probe/ slide (1ug=1ul)

**1 X SSC**

- 5 mls of 20X SSC
- 95mls of $H_2O$

**0.5 X SSC**

- 2.5mls of 20X SSC
- 97.5mls of $H_2O$

**10mM DTT in SSC**

- 1.2g DTT into 800mls SSC

**Ulysis ™Alexa Fluor ® 488 Nucleic Acid Labeling Kit**

- Component A: ULS Reagent
- Component B: DMSO
- Component C: Labeling Buffer
- Add 5µl Component B to one vial of Component A.
- Denature sense and anti-sense oligonucleotides separately at 95°C for 5 minutes and snap cool on ice. Centrifuge briefly to redeposit the sample to the bottom of the tube.
- Add 1µl of Component A with DMSO added per 1µg of DNA in new appropriate eppendorfer tubes. If necessary, use Component C to bring final volume of each tube to 25µl.
- Incubate both eppendorfer tubes (all components and oligonucleotides) at 80°C for 15 min. Stop reaction by plunging the reaction into ice bath. Centrifuge tube briefly to redeposit sample to bottom of the tube.

**EXPERIMENTAL PROTOCOL**

**Protocol Summary:**

1. **Tissue Preparation**
2. **In-situ hybridization with Ulysis ™ Alexa Fluor ® 488**
3. **Detection**

**Helpful Notes**:

A. Tissue Preparation: once tissue sections are rehydrated, do not let sections dry out at any step.

On the day before the experiment label, set up & fill 250 mL of solution into glass dishes as follows:

Deparaffinization:
3 dishes for Xylene
2 dishes for 100% EtOH
1 dishes for 95% EtOH
1 dish for 70% EtOH
1 dish for 50% EtOH
1 dish for DEPC $H_2O$
1 dish for PBS

1. Remove slides for storage and allow to warm to RT.
2. Arrange slides vertically into staining racks.
3. Dip staining rack into Xylene I dish for 2 min.; proceed to then dip rack into Xylene II for 2 min; proceed to then dip rack into Xylene III for 2 min.
4. Dip staining rack into 100% EtOH for 2 min; then dip rack into second 100% EtOH for 2 min.
5. Dip staining rack into 95% EtOH for 5 min.
6. Dip staining rack into 70% EtOH for 5 min.
7. Dip staining rack into 50% EtOH for 5 min.

8. Two quick dips of staining rack into DEPC $H_2O$.

9. Dip staining rack into DEPC-PBS I for 5 min; then dip rack into second DEPC-PBS II for 5 min.

10. Post-fix sections for 10-15 min with 4% PFA in 0.1M PB solution.

11. Wash slides with PBS for 5 min; wash again with PBS for 5 min.

12. Incubate slides for 20-30 min at 37 with 100-500ug/ml pepsin in 0.2M HCl. **OR** Incubate sections in 0.3% Triton X-100 for 15 min.

13. Wash slides in PBS for 5 min; wash again in PBS for 5 min.

14. Dispense 50 µL of warm (30 min in 37°C incubator) pre-hybridization buffer to each tissue section and incubate at 37°C for 2hrs in humid chamber.

15. Tap off pre-hybridization buffer and wash in 2X SSC for 5 min.

16. Tap off excuse SSC, circle tissue same with PAP Pen.

17. Dispense 50 µl of Alexa Fluor® 488 with respective oligonucleotides to appropriate tissue.

18. Carefully put your slides in a humid chamber for overnight incubation at 37C (approx 18 hrs). Hybridization can be left for up to 40 hours to increase signal intensity. DO NOT LET TISSUE DRY OUT. Carefully ensure all of your slides are kept level.

19. Prepare 0.5 and 1 X SSC solutions from 20X SSC and add DTT on day of use. (Note: make sure stock 0.5 and 1X SSC solution are at 55°C; 1.2 g DTT into 800 ml SSC=10mM)

20. Tap off hybridization solution and put slides into wash solutions.

| | | | |
|---|---|---|---|
| 21. | Quick Wash | 1X SSC (10mM DTT) | RT |
| 22. | 2 X 15mins | 1X SSC (10mM DTT) | 55°C |
| 23. | 2 X 15mins | 0.5 X SSC (10mM DTT) | 55°C |
| 24. | 1 X 10mins | 0.5 X SSC (10nM DTT) | RT |

25. Slides remain in last wash solution until the next step.

26. Begin to coverslip using glass rod to add 1-3 drops of Refrax mounting medium. Ensure there are no air bubbles.