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Introduction

Over half of all musculoskeletal disability is associated with degenerative disc disease (DDD), making this one of the most common and expensive medical conditions in the population, with low back pain representing the leading cause of disability in persons under the age of 45 [1-3]. The factors that account for the vulnerability of the disc to degeneration and the limited capacity of the disc for repair remain largely unknown. Moreover, there is currently no biological explanation for the discrepancy between individuals who do or do not develop DDD with age. Studies in twins have suggested that genetic susceptibility plays a critical role in the development of DDD [4, 5]. Some animal species, such as dogs, have naturally occurring strains that rarely develop DDD. These DDD-resistant strains are distinctive in that they maintain a resident population of notochord cells within the nucleus pulposus into adult life. Thus non-chondrodystrophic (NC) dogs preserve their notochord cells in the intervertebral disc and do not develop degenerative disease until very late in life [6-10]. In these animals, it has been demonstrated that coincident with the decline in the resident notochord cell population, the nucleus undergoes the internal disorganization and degeneration in the annulus fibrosis characteristic of DDD [6]. This temporal relationship suggests that the notochord cells may play a key role in disc homeostasis [6, 7, 11]. The important unanswered question is whether differential susceptibility to DDD seen in both dogs and humans is attributable to variable degrees of biochemical protection conferred by the local notochord cells in the disc. We have recently reported that supernatants of notochord cells obtained from NC canine disc nucleus pulposus have the ability to upregulate disc–derived chondrocyte proteoglycan production in a dose-dependent fashion [12]. This

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observed anabolic effect suggests that notochord cell-derived factors may be intrinsic to the physiology of the disc, and that differential resistance to DDD may be related to this relationship. The notochord cell may thus occupy a pivotal role in the maintenance of the matrix in the disc nucleus pulposus. Characterizing these notochord cell-derived growth factors may offer the new insights into innovative treatments for DDD in the future. In this study we characterize these factors and examine their effect upon the regulation of important genes in chondrocytes.

Results

Gross intervertebral disc morphology

We have previously described the gross appearance of the degenerative changes in the disc of chondrodystrophic and NC dogs [12, 13]. The histological appearance of the discs from these species shows that the NC canine nucleus is rich in notochord cells with modest cartilage matrix, whereas the chondrodystrophic animals have a disc nucleus that is sparse in notochord cells but rich in a dense cartilage matrix (Figure 1). It is noteworthy that such cells are characterized by their large size, highly vacuolated appearance and based upon buoyant density in Percoll density gradient [7, 10, 14]. There are as of yet no known specific cell surface markers for notochord cells, however their appearance is marked and characteristic. Our previous studies had demonstrated at least 6-fold difference in the resident NC cell population in the disc of the species [12, 13].

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Proteoglycan Gene Expression

Since large aggregating proteoglycans are critical to disc extracellular matrix (ECM) physiology, in particular load bearing and matrix interactions, we addressed whether chondrocytes cultured in NCCM demonstrate enhanced gene expression of aggrecan and versican, two such key proteoglycans. We also examined gene expression of hyaluronic acid synthase-2 (HAS-2) because of its role in the formation of hyaluronic acid, the long chain polymer to which proteoglycan aggregates attach by link proteins. Results of the gene expression experiments were consistent over 3 separate experiments using both notochord cells and chondrocytes from 3 different sets of animals. In these experiments, chondrocyte gene expression (mean) was upregulated 2.7-fold for aggrecan, 2.2-fold for versican, and 2-6 fold for HAS-2 (Figure 2) when cultured with NCCM as compared to DMEM only. Primer sequences and locus of the primers are given in Table 1.

Characterization of NCCM

Proteins obtained from NCCM were separated by SDS PAGE. The gel was stained with Colloidal Coomassie blue and the visualized bands were excised, digested with trypsin and subjected to LC-MS/MS mass spectroscopy. The spectra were evaluated with SONAR and MASCOT search engines. The peptides identified in NCCM were derived from: (i) Connective tissue growth factor (CTGF) precursor, (ii) Cu/Zn superoxide dysmutase, (ii) fibronectin, and (iv) tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) precursor. The peptide sequences and search scores obtained for the peptides are listed in Table 2.

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The canine genome was not completed at the time of our experiments therefore we verified the presence of the IGVCTAKDCAPCVFGGTVYR peptide in a protein-protein blast search on the NCBI database and selected the canine genome. The accession number for our search was XP_533406. Next we performed a search for nucleotide sequences for pig ctgf (query) matched against the canine genome (subject) using the locus as determined by the IGVCTAKDCAPCVFGGTVYR peptide (www.ebi.uniprot.org). The 3rd reading frame contains the canine ctgf sequence. Downstream from the sequenced peptide to the c-terminus there is extremely high homology. The only match for sequences showing significant alignments was connective tissue growth factor. The pig and canine genes were then compared for nucleotide homology using the EBI-Uniprot database and were found to be highly homologous differing only in a g-c substitution at position 56. We were careful to include the sequenced part of the gene within the amplicon generated by our RT-PCR experiments.

CTGF Gene Expression in Chondrodystrophic and NCD dogs

With the characterization of CTGF in NCCM we sought to address whether notochord cells from chondrodystrophic and NC dogs differentially expressed CTGF. Notochord gene expression of CTGF analyzed by RT-PCR was found to be comparable between the two canine species (Figure 3).

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Effects of rCTGF on Aggrecan Gene Expression

With the results of the mass spectroscopy, CTGF constituted a strong candidate for the anabolic properties of NCCM. We thus examined the effects of recombinant CTGF (rCTGF) on aggrecan gene expression in chondrocytes. Aggrecan is the dominant proteoglycan responsible for the hydrophilic and load-bearing characteristics of the disc and is known to be associated with CTGF anabolic activity. We observed that rCTGF recapitulated the effect of NCCM on chondrocyte aggrecan expression, with a dose-dependent relationship between rCTGF and aggrecan gene expression (Fig. 4). In terms of bioactivity, the effect of NCCM was comparable to 100-200ng/mL of rCTGF.

Discussion

NC dogs are relatively resistant to DDD compared to chondrodystrophic strains, but the reasons for these differences have been previously unresolved [15, 16]. The salient difference between these strains as observed in our studies and others however is the maintenance of the notochord cell population within the intervertebral disc nucleus of NC strains [7-10, 17, 18]. Aguiar et al previously suggested that notochord cells may produce trophic factors capable of stimulating chondrocyte proteoglycan production within the intervertebral disc, but the identity of such factors has remained unknown [7]. It has also been suggested that the notochord cells themselves may contribute enhanced physical characteristics to the disc [10]. Therefore maintenance of a notochord cell-rich nucleus might afford the disc optimal biophysical properties, highlighting this as an important area of study with respect to potential therapeutic interventions for DDD [10].

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This is the first demonstration that the disc-derived chondrocyte expression of aggrecan, versican and HAS-2 are upregulated by notochord cell-derived factors. Aggrecan is the primary proteoglycan responsible for load bearing within the disc [6, 17, 19-21]. Aggrecan and versican bind to hyaluronan and are stabilized by link proteins to the long hyaluronan polymers. In addition to load bearing, versican is known to confer important ECM interaction within the disc matrix [22]. The upregulation of HAS-2 further supports the notion that notochord cell-secreted factors specifically enhance proteoglycan constituents of the disc nucleus extracellular matrix.

Notochord cells have not previously been known to produce CTGF, a member of the CCN gene family. The current nomenclature reflects a number of related genes termed *ccn1* (cef-10/cy*r*61), *ccn2* (*ctgf/hcs24/fisp12*), *ccn3* (*nov*) plus several related genes including ccn4 (*elm-1/wisp-1*), *ccn5* (*ctgf-3/wisp-2/cop1*) [23, 24]. CTGF is a cysteine-rich secretory protein of 36-38 kDa containing 349 amino acid residues. CTGF contains a von Willebrand type C domain that interacts with growth factors such as TGF-β and BMPs, and thereby mediates ECM interactions [24, 25]. The activity of CTGF varies in a tissue-specific way. With respect to connective tissues and cartilage in particular, CTGF activity may be modulated by both TGF-ß and BMP-2, both of which are involved with bone and cartilage formation [26-29]. The interaction of CTGF with TGF-β and BMP is important in that CTGF inhibits BMP signaling but can mediate enhanced signaling by relatively low levels of TGF-β1 [26, 30, 31]. The CTGF promoter contains a TGF-β response element further implicating a relationship between members of the TGF-β family and CTGF. It has been reported that increased expression of the

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CTGF/Hcs24 gene in HCS-2/8 chondrocytic cells is the result of high transcriptional activity of the CTGF/Hcs24 promoter suggesting that the TGF-β response element is critical to the support of such high transcription activity [30]. Kireeva et al reported that fibroblast DNA synthesis was not stimulated directly by CTGF however the activity of other growth factors such as bFGF was augmented when cultured in the presence of CTGF, thus emphasizing the additive effect of CTGF with other growth factors [32]. A synergistic action of CTGF with TGF-ß leads to protracted preservation of ECM in the mouse when compared to TGF-ß or CTGF alone [32]. CTGF alone is capable of independently inducing matrix synthesis, reflecting its function as an auto-inducer [33-35].

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CTGF has been described as having both apoptotic as well as anti-apoptotic properties [23, 27, 36-39]. It is known that CTGF is an auto-inducer in many cells therefore inhibition of this autocrine pathway and subsequent apoptotic changes strongly suggests that CTGF has anti-apoptotic functions in some cells. Using human aortic smooth muscle cells that CTGF induces apoptosis via the caspase-3 pathway [40]. Nakanishi et al reported that aggrecan, collagen type II and X and alkaline phosphatase were stimulated in chondrocytes treated with rCTGF in a dose-dependent fashion [41], similar to our observations with chondrocytes from the intervertebral disc. Since CTGF is known to promote ECM synthesis and inhibit apoptosis, this factor in the disc nucleus may figure prominently in the resistance of certain species to the development of DDD. We have demonstrated that notochord cells from chondrodystrophic and NC dogs demonstrate comparable CTGF gene expression suggesting that quantitative differences in the

persisting NC cell population may be the critical difference between these species. It has been speculated that once the disc has developed, the notochord cells eventually disappear through apoptosis [42]. However to date there is no explanation for the persistence of notochord cells in the nucleus of NC dogs as compared to the paucity of such cells in the chondrodystrophic animals. The role played by CTGF within the disc or its effect upon both the notochord cells themselves, the resident chondrocytes and the ECM has not been previously addressed. Thus recent studies support the notion that CTGF may play a critical role in chondrocyte biology, both organizational in homeostatic and anabolic activities, possibly in concert with other growth factors. Consistent with the hypothesis that CTGF may be a vital factor in ECM homeostasis within the intervertebral disc, Nishida et al found that hydrogels containing rCTGF/CCN2 implanted into articular cartilage defects in rat knees led to the development of new cartilage that was histologically indistinguishable from normal cartilage [28].

With respect to the pivotal role played by CTGF in tissue differentiation and organization, a recent study examining comparative genomics and midline expression profiling in zebrafish and pufferfish reported that a conserved region overlaps the first exon of the CTGF gene and is involved with floor plate differentiation in the developing embryo [43]. The floor plate shares sequence similarity with the notochord in terms of sharing a likely promoter, the *up1* region that overlaps exon1 of the CTGF gene. This is the first time that CTGF has been reported to be associated with the developing embryonic notochord. It was also noted that insertion of the *up1* promoter in a downstream position from the reporter transcription site still resulted in changes of

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midline expression in zebra fish, leaving the question of the precise mechanism of this conserved region in question [43]. However the shared conserved region with the CTGF gene strongly suggests important organizational characteristics on the part of this gene. Since CTGF is known to promote ECM synthesis, proteoglycan production, collagen production and interacts with other growth factors, it is possible that the presence of this factor in the disc nucleus may figure prominently in the resistance of certain species to the development of DDD. We are aware that there are likely other candidate growth factors within the NCCM, however we were only able to sequence and identify CTGF thus far. There are a number of members of the CTGF family, inclusive of isoforms that may or may not also be present within NCCM. Therefore we chose to examine the effects of rCTGF to evaluate its capacity to reproduce our observations of aggrecan upregulation by NCCM.

We are aware that the use of semi-quantitative RT-PCR has some inherent limitations with respect to sensitivity and reliability as compared to real-time methods. We did not have access to real-time RT-PCR at the time of these studies and plan to further examine the effects of NCCM and rCTGF upon chondrocytes using these methods in current and future studies. However, very strong trends of upregulation of the genes studied (particularly for aggrecan-repeated 9 times) strongly indicate that our observations are correct.

Our results suggest that non-chondrodystrophic canines are spared the development of DDD because their discs contain an abundance of notochord cells that secrete a key anabolic factor, CTGF. We have demonstrated that the expression of CTGF is comparable between the NCD and CD canine notochord cells, suggesting that the critical

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difference between these two species may be the larger population of notochord cells in the nucleus pulposus of animals resistant to DDD. These findings provide insight into the biology of the intervertebral disc and raise the possibility of future novel therapeutic options for this disabling condition.

Methods and Materials

Chondrodystrophic and NC Canine Discs

Animals were obtained in collaboration with the University of Guelph, School of Veterinary Medicine. All dogs were 8-12 months of age.

Chondrocyte Culture Conditions for Gene Expression Studies

Intervertebral disc chondrocytes were obtained by dissecting the nucleus pulposus from fresh bovine caudal discs and placed in alginate bead cultures as previously described using Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin, streptomycin and fungizone (PSF) and 10% fetal calf serum (FCS) during the overnight enzymatic period only[13]. Thereafter 100 alginate beads containing the chondrocytes were cultured in wells and maintained in serum-free conditions (DMEM/PSF) for 4 days with daily changes of serum-free medium. The medium was then discarded and was replaced by variable doses of 50, 100 or 200 ng/mL of rCTGF or by NCCM obtained from the notochord cell cultures. DMEM was used in parallel as a negative control. These cultures were maintained for 24 hr at 37^0C in a humidified incubator at 5% CO_2. Total RNA was extracted from the treated and untreated chondrocytes using Trizol and quantified using spectrophotometric assay at $OD_{260/280}$.

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Generation of Notochord-Cell Conditioned Medium (NCCM)

NCCM was developed from 6 separate sources of 5 animals. Within 2 hours of euthanasia of the animals, the lumbar spines were removed, the soft tissues dissected away from the spine and the spines washed with DH20, Clidox ™, and liberally covered with Betadine™. Under aseptic conditions the nucleus pulposus was removed from each of 6 lumbar discs and placed in DMEM containing 100U penicillin/streptomycin. For histology, vertebra-disc units were fixed in 4% formalin for 3 days and then processed through graded xylenes and alcohols, mounted and stained with Toluidine Blue. The intervertebral disc nucleus pulposus was removed from 6 lumbar segments of each animal and the nucleus pulposus tissues were rinsed with 150 mM NaCl containing (PSF) and sequentially digested overnight using pronase and collagenase [7, 44]. The cells were recovered from a Percoll density gradient [7, 12]. The cells were freed from Percoll and after assessment for viability they were placed in alginate bead culture (120 beads/well at a density of approximately 1 x 10^6 cells/ml for 4 days in a total volume of 2.5 ml of DMEM containing PSF but no fetal calf serum. Tissue culture conditions were 37^0C in 5% CO_2. The medium was removed and filtered through a 0.2 μm syringe-tip filters as NCCM.

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Gene Expression Studies

We examined gene expression of aggrecan, versican and HAS-2 (primer sequences Table 1). 1 μg of total RNA was reverse transcribed followed by PCR for each gene studied using RNA extracted from both treated and untreated cells. The number of PCR cycles used for each gene was between 18 and 35 cycles under the same PCR parameters, except

for annealing temperatures which were as follows: 60^0C for HAS-2, 62^0C for aggrecan, 64^0C for versican, 62^0C for HPRT, and 55^0C for CTGF. PCR amplicons were electrophoresed on a 1.7% agarose gel containing 0.05% ethidium bromide with TBE buffer and evaluated using volume-based densitometry with the Bio-Rad 'Gel Doc' imaging system. Net density of each amplicon was normalized to the housekeeping gene HPRT and expressed as a ratio. All amplicons were evaluated and plotted to ensure that the cycles studied fell within the linear region of the amplification curve. Each gene studied was examined at least in triplicate.

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Mass Spectroscopy Analysis of NCCM

LC-MS/MS mass spectroscopy was used to identify the amino acid sequences of the proteins recovered from the NCCM. After a 4-day tissue culture period the NCCM was harvested from the tissue culture plates, filtered twice using 0.2μm syringe-tip filters, and the supernatant was precipitated overnight using ice-cold absolute ethanol diluted to 95%. A series of repetitive precipitation steps using EtOh, re-suspension in 0.03M Tris-Cl (pH 6.8) and HPLC-grade DH_20 were undertaken with centrifugation at 13,000 X G for 20 min at each step. The last step consisted of NCCM dissolved in HPLC-grade DH_20 and a final centrifugation of 13,000 X G for 30 min. The supernatant was then removed and subjected to SDS-PAGE purification on a 10% and a 4-20% Tris-glycine gel and stained with Gel Code Colloidal Coomassie Blue (Pierce). The gel was fixed for 70 min with 10% glacial acetic acid and 50% methanol and the image captured using the ProXPRESS gel imager (Perkin Elmer).

Visualized bands after staining were excised, diced into ~1mm cubes and placed into a 96 well microtitre reaction plate for tryptic digestion in the ProGEST robot (Genomic Solutions Inc.). After digestion, the bands were subjected to LC-MS/MS mass spectroscopy and the MS/MS spectra were compiled and analyzed using SONAR (Proteometrics Inc., Winnipeg, Manitoba, Canada) and/or MASCOT (Matrix Science Ltd., London UK) search engines.

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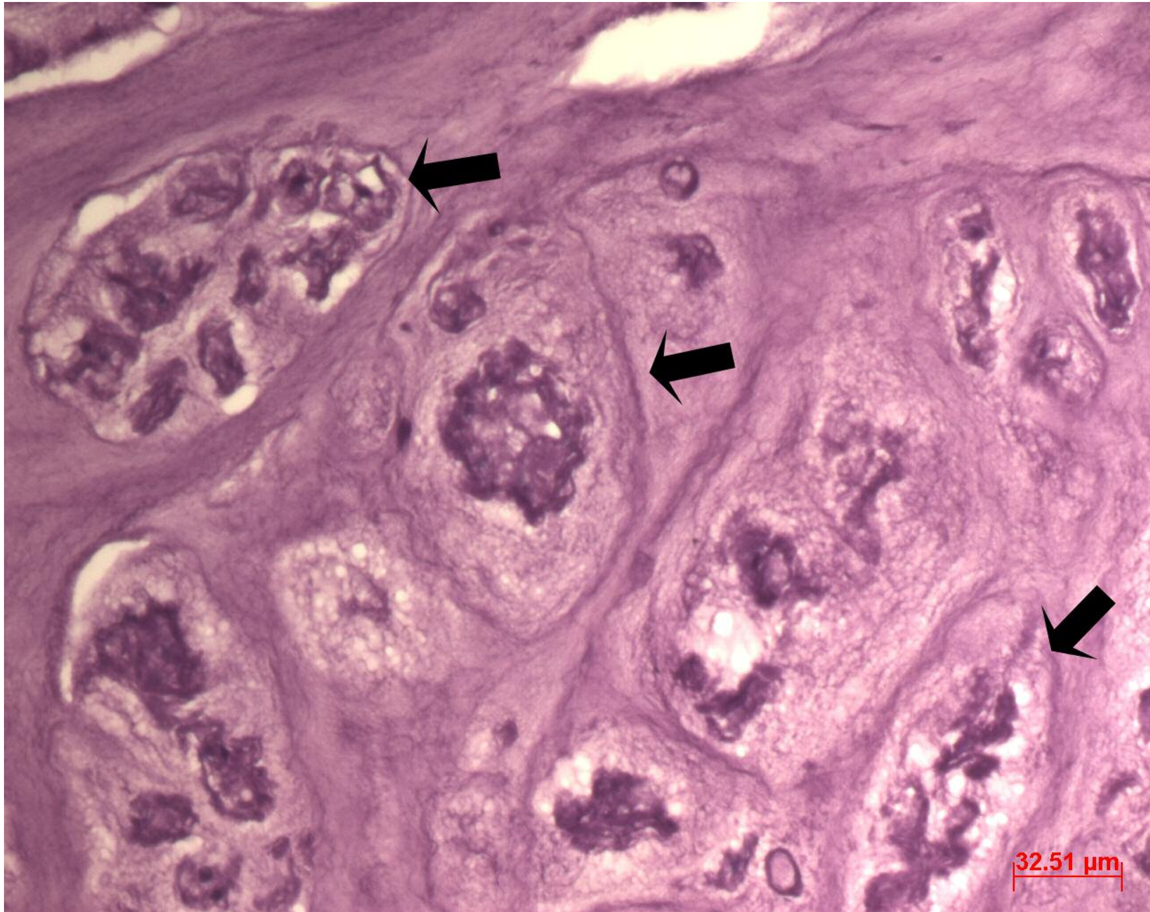

Figure 1 (a) x 20 magnification of the centre of non-chondrodystrophic, nucleus pulposus (toluidine blue). This photograph depicts the amorphous appearance of the nucleus with some cartilage matrix and an abundance of physaliferous notochord cells. Arrows show typical notochord cells. There was always some distortion of cytoarchitecture when fixing notochord cell-rich tissues to tendency of the extracellular matrix to swell to a great degree.

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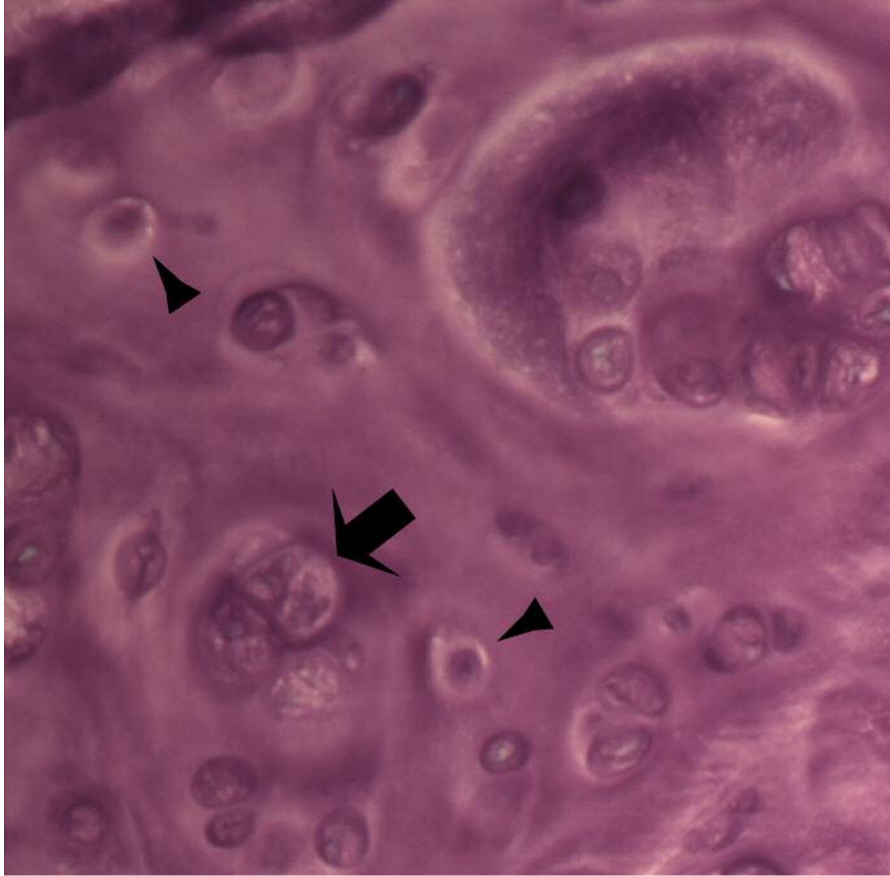

Figure 1 (b) x 20 magnification of the centre of a chondrodystrophic canine intervertebral disc nucleus pulposus demonstrating and abundance of chondrocyte-like cells and matrix but sparce evidence of notochord cells (Toluidine Blue). Chondrocytes are shown by arrowheads, notochord cells by arrow.

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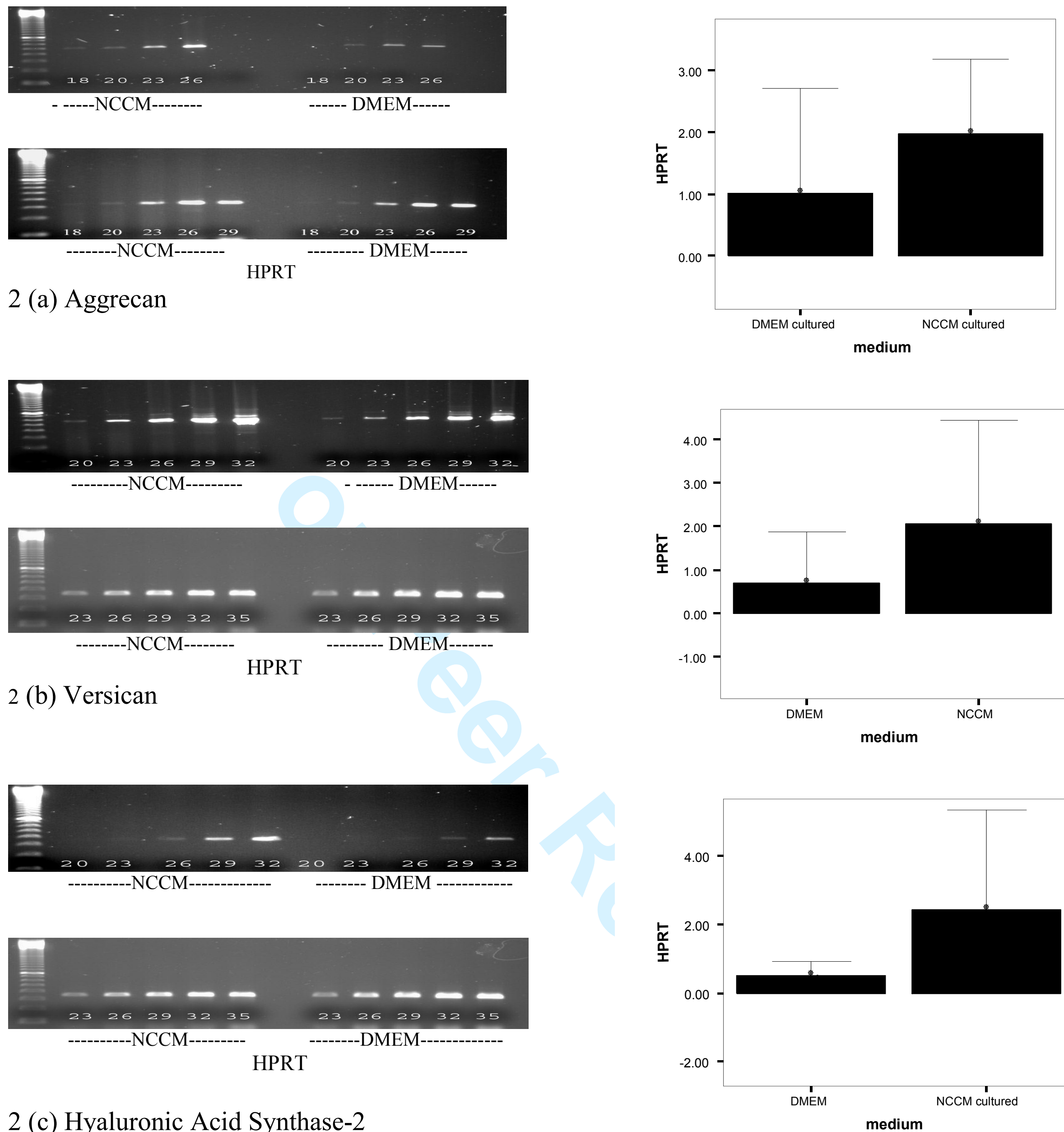
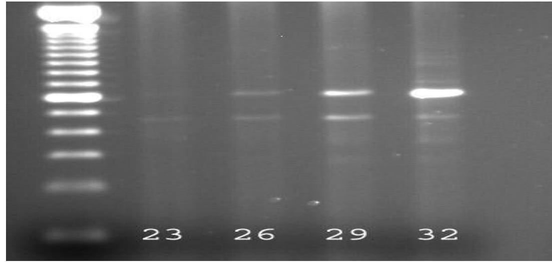


2 (c) Hyaluronic Acid Synthase-2

Figure 2 (a-c): The above are representative examples of aggrecan, versican and hyaluronan synthase-2 gene expression studies (1.7% Agarose Gels, 0.5% Ethidium Bromide). A range of PCR cycles were examined in order to determine the linear aspect of the amplification curve from which appropriate PCR cycles were evaluated. All amplicons obtained by PCR were analyzed for net amplicon density (Bio-Rad Gel Doc system) and were normalized to the housekeeping gene HPRT and expressed as a ratio. Figure 3 (a, b, and c) depict amplicons generated from aggrecan (PCR cycle 23), versican (PCR cycle 23) and hyaluronan synthase-2 (PCR cycle 29) primers. Aggrecan is upregulated 2.7 fold P=.028. Versican is upregulated 2.2 fold P=.028 and hyaluronic acid synthase-2 is upregulated 2.6 fold P=.009. All data was analyzed using SPSS statistical software version 12.1 and a two samples t-test. The gels represented above depict a minimum of three separate experiments using different sets of notochord cells and chondrocytes.



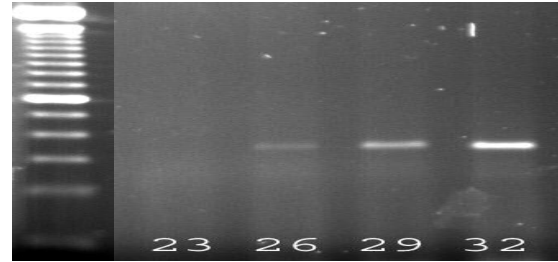
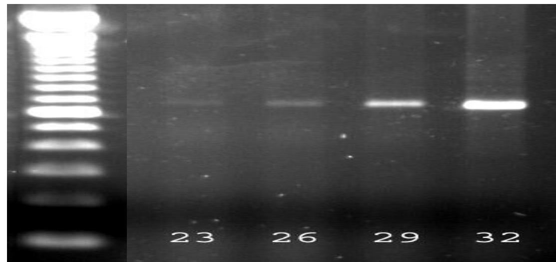


Figure 3 (a) Non-chondrodystrophic canine notochord cells (b) HPRT



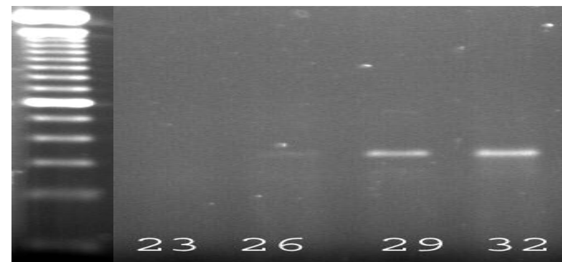


Figure 3 (c) Chondrodystrophic canine notochord cells (d) HPRT

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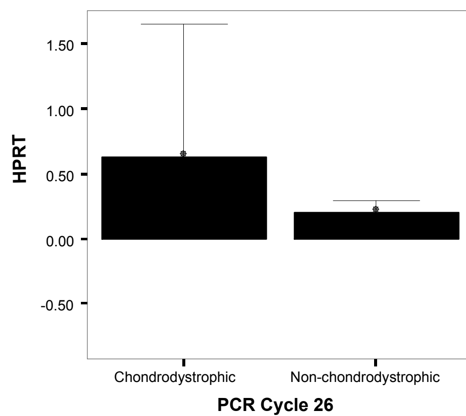

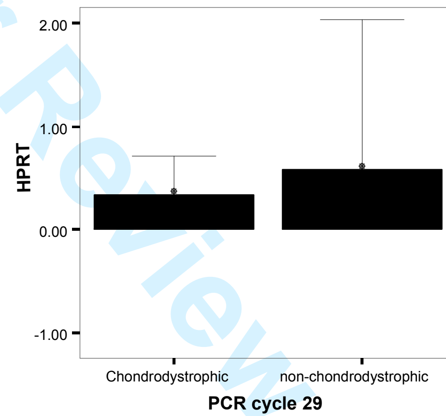

Figure 3 (e)

Figure 3 (a-d): CTGF gene expression by non-chondrodystrophic and chondrodystrophic canine notochord cells. (e) CTGF gene expression ratios examined at PCR cycles 26 and 29. There is no difference in CTGF gene expression between the two sub-species of dog.

Gene	Primer sequence
Aggrecan (G3 domain)	5' GAC AGA TGA TTC AGA GGC AAC
	3' CAG GGC ATT GAT CTC GTA TC
Primer Location	bp 6669-7164
Versican (V3) (G3 domain)	5' CTC ACC AGT ATC CTG TCT CAC
	3' CGT TCT TAG TTC CGA GAC TAG
Primer Location	Bp 1475-2060
Hyaluronan synthase-2	5' CTT AGA GGA AAC ATT GTC ATG G
	3' CGT AAG ATC ATA CAT CAA GCA C
Primer Location	bp 1822-2210
HPRT	5' CTC ATG GAC TAA TTA TGG ACA
	3' TAC GTC TGA AAC GAA CGG AAC
Primer Location	bp 71-558
Connective Tissue Growth Factor	5' CTG CCC AGC CCC GAC TGC 3' GCT TTA CGC CAT GTC TCC ATA
Primer Location	bp 632-1258

Table 1: PCR primer sequences and locus of primer within each gene

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Peptides Sequenced	Peptide locus	Accession No.	Sonar Score	Mascot Score
IGVCTAKDCAPCVFGGTVYR **Connective tissue growth factor precursor (CTGF)** Sus Scrofa	92-111	NP_998998	8.6 x 10^{-5}	43
FVDTPALESVCGYLHR DGHLQINTCSFVAPWSSLSTAQR **Tissue inhibitor of matrix metalloproteinase-1** (canis familiaris)	83-98 114-136	BAA32393		71
DDLGKGDNEESTQTGNAGSR AHVGDLGNVTAGKDGVAIVSIEDSLIALSGDY **Cu/Zn superoxide dysmutase** (canis familiaris)	124-143 80-115	Q8WNN6		204
SYTITGLQPGTDYK FLATTPNSLLVSWQPPR **Fibronectin** (Canis Familiaris)	80793 124-140	AAC48611	4.0 x 10^{-30}	140

Table 2: Peptide sequences obtained by MS/MS fragmentation of NCCM. Loci of peptide sequences and accession numbers are given for the identified peptides as well as the scores for each peptide sequenced. Peptides were searched with both MASCOT and SONAR search engines. The MASCOT search engine ranks a score of .43 as identity or extensive homology ($p<.05$) whereas SONAR provides for a statistical likelihood that the match is a random event. The smaller the SONAR score, the smaller statistical likelihood of a random match.

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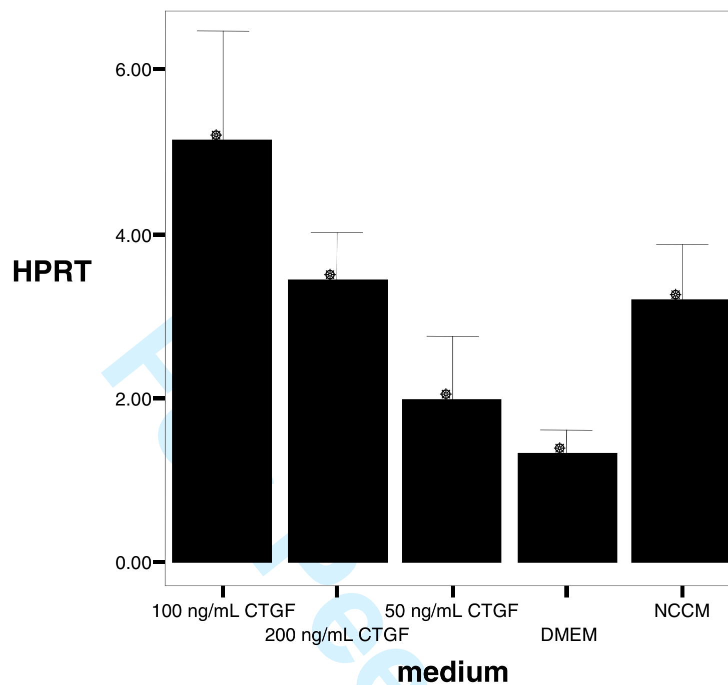

Figure 4: Aggrecan gene expression (normalized to HPRT) for disc-derived chondrocytes. Chondrocytes were cultured for 24 hours with either DMEM, 50ng/ml, 100ng/ml, 200 ng/ml rCTGF and NCCM. Amplicons were generated from the reverse transcription and subsequent PCR using aggrecan specific primers of 1μg total RNA harvested from treated chondrocytes (Trizol). The above results reflect mean gene expression ratios of between 5 and 9 separate experiments all from at least 3 separate sources of chondrocytes and notochord cells. DMEM and CTGF 50 were repeated 3 times, CTGF 100 and 200 were repeated 8 times, and NCCM was repeated 9 times.

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