Effects of interleukin-6 and interleukin-1β on expression of growth differentiation factor-5 and Wnt signaling pathway genes in equine chondrocytes

Emilia Svala, MSc; Anna I. Thorfve, MSc; Cecilia Ley, DVM, PhD; Helena K. Barreto Henriksson, PhD; Jane M. Synnergren, PhD; Anders H. Lindahl, MD, PhD; Stina Ekman, DVM, PhD; Eva S. R. Skiöldebrand, DVM, PhD

Objective—To determine the effects of interleukin (IL)-6 and IL-1β stimulation on expression of growth differentiation factor (GDF)-5 and Wnt signaling pathway genes in equine chondrocytes.

Sample—Macroscopically normal articular cartilage samples from 6 horses and osteochondral fragments (OCFs) from 3 horses.

Procedures—Chondrocyte pellets were prepared and cultured without stimulation or following stimulation with IL-6 or IL-1β for 1, 2, 12, and 48 hours; expression of GDF-5 was determined with a quantitative real-time PCR assay. Expression of genes in various signaling pathways was determined with microarrays for pellets stimulated for 1 and 2 hours. Immunohistochemical analysis was used to detect GDF-5, glycogen synthase kinase 3β (GSK-3β), and β-catenin proteins in macroscopically normal cartilage samples and OCFs.

Results—Chondrocytes stimulated with IL-6 had significantly higher GDF-5 expression within 2 hours versus unstimulated chondrocytes. Microarray analysis of Wnt signaling pathway genes indicated expression of GSK-3β and coiled-coil domain containing 88C increased after 1 hour and expression of β-catenin decreased after 2 hours of IL-6 stimulation. Results of immunohistochemical detection of proteins were similar to microarray analysis results. Chondrocytes in macroscopically normal articular cartilage and OCFs had immunostaining for GDF-5.

Conclusion and Clinical Relevance—Results indicated IL-6 stimulation decreased chondrocyte expression of the canonical Wnt signaling pathway transactivator β-catenin, induced expression of inhibitors of the Wnt pathway, and increased expression of GDF-5. This suggested IL-6 may inhibit the Wnt signaling pathway with subsequent upregulation of GDF-5 expression. Anabolic extracellular matrix metabolism in OCFs may be attributable to GDF-5 expression. This information could be useful for development of cartilage repair methods. (*Am J Vet Res* 2014;75:132–140)

Received August 1, 2013.
Accepted October 3, 2013.

From the Department of Biomedical Sciences and Veterinary Public Health, Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden (Svala, Ley, Ekman, Skiöldebrand); the Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Sahlgrenska University Hospital (Henriksson, Lindahl), and the BIOMATCELL, VINN Excellence Center of Biomaterials and Cell Therapy at the Department of Biomaterials, Institute of Clinical Sciences (Thorfve), University of Gothenburg, 411 37 Gothenburg, Sweden; and System Biology Research Centre, School of Humanities and Informatics, University of Skövde, 541 28 Skövde, Sweden (Synnergren). Drs. Svala and Skiöldebrand's present address is Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Sahlgrenska University Hospital, University of Gothenburg, 411 37 Gothenburg, Sweden. Dr. Henriksson's present address is Department of Orthopaedics, Institute of Clinical Sciences, Sahlgrenska University Hospital, University of Gothenburg, 411 37 Gothenburg, Sweden.

Supported by grants from the Swedish-Norwegian Foundation for Equine Research (H0947014) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas 2006-1947).

The authors declare that they have no conflicts of interest.

Address correspondence to Dr. Svala (emilia.svala@slu.se).

Abbreviations

BMP	Bone morphogenetic protein
CCDC88C	Coiled-coil domain containing 88C
COMP	Cartilage oligomeric matrix protein
FZD	Frizzled family receptor
GDF	Growth differentiation factor
GSK	Glycogen synthase kinase
IL	Interleukin
LRP	Lipoprotein receptor-related protein
OA	Osteoarthritis
OCF	Osteochondral fragment
PPARD	Peroxisome proliferator-activated receptor delta
qRT-PCR	Quantitative real-time PCR

Osteochondral fragments, also known as chip fractures, of the dorsal aspects of the articular margins and facets of bones in the carpal joints and the proximal aspect of the first phalanx can develop in racehorses with OA. Osteochondral fragments are thought to develop in degenerated articular cartilage and necrotic subchondral bone or from fractured osteophytes.[1]

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

The metabolic activity in carpal joints with OCFs is higher than it is in joints without OCFs, as indicated by results of another study[2] that aggrecan and COMP are upregulated in such joints. Such increased activity may be attributable to metabolic characteristics of the OCFs, which could be induced by the inflammatory cytokine IL-6. High concentrations of this cytokine have been detected in synovial fluid samples collected from joints with OCFs,[3] and chondrocytes of OCFs are a potential cellular source of IL-6.[4]

In osteoarthritic joints, there is an imbalance in extracellular matrix turnover with high catabolic activity induced by IL-1β and tumor necrosis factor-α[5]; IL-1β induces production of other cytokines such as IL-6[6] and IL-8[7] that may amplify and sustain the inflammatory response. However, results of another study[8] indicate that these cytokines have a modulatory or an inhibitory effect similar to that of IL-4, IL-10, and IL-13.[8] Moreover, IL-6 gene knockout mice develop severe OA, suggesting that cytokine has a protective role in cartilage.[9] In addition, IL-1β upregulates IL-6 mRNA expression in equine chondrocytes grown in monolayer[10] and pellets.[11]

Growth differentiation factor-5, also known as cartilage-derived morphogenetic protein 1 and BMP-14, is a member of the transforming growth factor β superfamily. Growth differentiation factor-5 stimulates chondrocyte metabolic activity and chondrogenesis in vitro and in vivo and has been detected in human cartilage obtained from clinically normal and osteoarthritic joints[12,13] and bone.[14] The BMPs, including GDF-5, have a role in cartilage and bone formation during embryogenesis; reactivation of these signaling pathways has been proposed to be an important part of postnatal joint repair and homeostasis.[14–17] Several pathways have been implicated in regulation of GDF-5 expression, including the Wnt signaling pathway (named for *Drosophila melanogaster* wingless mutant integration site [wingless-type mouse mammary tumor virus integration site family {*WNT* genes}]), which is required for induction of GDF-5 expression in the joint interzone.[18] Signaling through the Wnt pathway is dependent on Wnt ligands that bind to FZDs. With regard to the canonical Wnt signaling pathway, the coreceptor LRP-5/6 is also required for transduction of the signal, and several secreted antagonists regulate Wnt signaling. Activation of FZDs and LRP-5/6 triggers phosphorylation of dishevelled homolog proteins, disrupting the β-catenin degradation complex containing GSK-3β. This complex typically degrades β-catenin by means of proteasomal degradation; however, when Wnt ligands bind FZDs, cytosolic β-catenin accumulates and is translocated into the cell nucleus, where it induces expression of canonical Wnt signaling pathway target genes such as cyclin D1, PPARD, and fos-related antigen 1.[19–21]

Results of another study[11] by personnel in our laboratory suggest that 48 hours of stimulation of chondrocytes with IL-6 can induce mild prochondrogenic effects at the gene level, whereas antichondrogenic effects are detected after IL-1β stimulation. The hypothesis for the study reported here was that IL-6 would induce upregulation of GDF-5 in equine OCFs obtained from joints with OA. To test this hypothesis, we determined the in vitro effects of IL-6 and IL-1β on GDF-5 gene expression in a 3-D equine chondrocyte pellet culture system. We also performed global transcriptional analysis with microarrays after short-term (1 and 2 hours) IL-6 stimulation of equine chondrocyte pellets to identify the signaling pathways involved in IL-6–mediated upregulation of GDF-5 expression.

Materials and Methods

Samples—Macroscopically normal cartilage samples were obtained from middle carpal joints of 6 horses (mean age, 1.9 years; age range, 0.5 to 3 years) without known clinical history of disease in those joints. Cartilage samples were obtained within 24 hours after euthanasia. Horses were euthanized because of reasons unrelated to this study. Study protocols were approved by the Ethical Committee on Animal Experiments, Stockholm, Sweden. Following aseptic preparation, arthrotomies were performed, cartilage of the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel, and full-thickness cartilage samples were collected with forceps. Shavings of cartilage were pooled from left and right joints and placed in sterile saline (0.9% NaCl) solution with gentamicin sulfate (50 mg/L) and amphotericin B (250 µg/mL). Cartilage samples were transported chilled (approx 5°C) to the laboratory. Isolation, expansion, and 3-D culture of chondrocytes were performed as previously described.[11] Culture media was changed daily after chondrocyte pellets were established.

Following collection of cartilage samples, 2- to 3-mm osteochondral samples were obtained from adjacent sites by cutting with a band saw[a]; osteochondral samples were then placed in neutral-buffered 10% formalin. Osteochondral samples were decalcified in 3.4% (wt/vol) sodium formate and 15.1% (vol/vol) formic acid, dehydrated, embedded in paraffin, and sectioned (thickness, 6 µm). The osteochondral samples included sections of articular cartilage that comprised all zones of hyaline articular cartilage and calcified cartilage (separated by the tidemark) and subchondral bone.

Osteochondral fragments of the dorsoproximal aspect of the radial facet of the third carpal bone, dorsodistal aspect of the radial carpal bone, or dorsoproximal aspect of the first phalanx were collected from 3 horses (ages, 3, 4, and 10 years) that underwent arthroscopy because of joint problems that caused lameness. All of these joints had additional articular cartilage lesions, such as fibrillation, that indicated OA. The OCFs included articular cartilage with areas of moderate surface fibrillation. The OCFs were placed in neutral-buffered 10% formalin, decalcified, embedded in paraffin, and sectioned (thickness, 6 µm). Disorganized cellular zones included rounded chondrocytes in clusters and adjacent trabecular bone (determined by means of light microscopy).

Cytokine stimulation of chondrocyte pellet cultures—On day 14 of culture, chondrocyte pellets obtained from each of the 6 horses were allocated with a randomization procedure to 3 groups. Chondrocyte pellet cultures were stimulated with recombinant equine IL-1β[b] (5 ng/mL) or recombinant equine IL-6[b]

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

(5 ng/mL) or were not stimulated.[11] Pellets from 4 of the horses were stimulated for 1 and 2 hours (short-term stimulation). Pellets from 3 of the horses were stimulated for 12 hours, and pellets from 4 of the horses were stimulated for 48 hours (long-term stimulation). At the end of the stimulation period, chondrocyte pellets were fixed in neutral-buffered 6% formalin and embedded in paraffin for evaluation by means of light microscopy or washed in PBS solution, snap frozen in liquid nitrogen, and stored at –80°C for gene expression analysis.

Isolation of total RNA and cDNA synthesis—Chondrocyte pellets (duplicate pellets for each treatment group) were disrupted in 1.5-mL polypropylene tubes with a tungsten bead[c] for 10 minutes by use of a homogenizer.[c] Chondrocyte pellet samples were frozen repeatedly in liquid nitrogen during the procedure. Total RNA was extracted with a lysis reagent and chloroform, then purified with a commercially available kit[c] in accordance with the manufacturer's protocol for animal cells. Genomic DNA was removed[c] from the isolated RNA, and 100 ng of total RNA for each chondrocyte pellet sample was reverse-transcribed[d] to cDNA.

qRT-PCR assay—A database[e] and genome browser[f] were used to select equine-specific primer and probe[d] sequences for beta glucuronidase, which was used as a qRT-PCR assay reference gene[11] (forward primer, 5′-GTGACCAACTCCAACTATGAAGCA-3′; reverse primer, 5′-AGGAGTAGTAACTATTCACACAGATGACA-3′; probe, 6-carboxyfluorescein-CATATGGCGCCCCTAGGTC-dihydrocyclopyrroloindole tripeptide minor groove binder). A commercially available qRT-PCR assay mix of primers and probes intended for detection of human genes was used for detection of equine GDF-5.[d] A basic local alignment search tool[g] was used to verify homology between human and equine genes. The qRT-PCR assay was performed by use of a real-time PCR assay system[d] with a solution of polymerase, nucleotides, and buffer.[d] Relative gene expression was determined with the $2^{-(\Delta\Delta CT)}$ method[22] with qRT-PCR data analysis software.[h]

Global transcriptional analysis with microarrays—The RNA quality was analyzed,[i] and the concentration was measured with a spectrophotometer.[j] Total RNA (250 ng) of chondrocyte pellets (n = 4 horses) that were stimulated with IL-6 (5 ng/mL) or unstimulated for 1- and 2-hour stimulation times were processed and analyzed[k] with a microarray[l] for global transcriptome quantification intended for detection of human genes in accordance with the manufacturer's protocol. The high-throughput array was scanned, and expression signals were extracted and normalized by use of the robust multichip average normalization method. The data were used to screen for putative target genes that had a change in expression as indicated by comparison of results for IL-6–stimulated chondrocyte pellets with those for unstimulated pellets. A Web-based probe match tool[m] was used to compare equine nucleotide sequences to those of probes for human genes on the array to confirm that human probe sequences matched the query sequences for corresponding equine genes.

Immunohistochemical analysis—For GDF-5 immunostaining, sections of selected samples of macroscopically normal articular cartilage and OCF samples were deparaffinized and permeabilized with a detergent solution[n] for 10 minutes; blocked for nonspecific binding with 2% bovine serum albumin, 0.1% detergent solution, and 100mM glycine for 15 minutes; and incubated with goat polyclonal GDF-5 antibody[o] (diluted 1:200) at 4°C overnight (approx 15 hours). Additional blocking to avoid nonspecific binding was performed before incubation with fluorescent secondary donkey anti-goat antibody.[d]

For β-catenin and GSK-3β immunostaining, sections of unstimulated chondrocyte pellets and pellets stimulated with IL-1β and IL-6 for the 1 hour stimulation time were treated with 3% hydrogen peroxide before antigen retrieval with 10mM sodium citrate buffer for 30 minutes at 98°C. Sections were rinsed in 100mM Tris, saline solution, and 0.1% detergent buffer; blocked for nonspecific binding; and incubated with the primary antibodies mouse monoclonal nuclear dephosphorylated β-catenin[o] (diluted 1:200) or rabbit polyclonal GSK-3β[o] (diluted 1:100) at 4°C overnight. Sections were washed and blocked again before incubation with goat anti-rabbit IgG horseradish peroxidase conjugate antibody[o] (diluted 1:150) or goat anti-mouse IgG horseradish peroxidase conjugate antibody[p] (diluted 1:150) for 2 hours at room temperature.

Primary antibodies were detected by use of a signal amplification kit[q] (dilution, 1:150). Isotype-specific immunoglobulins (normal rabbit IgG, normal rabbit immunoglobulin, or normal mouse IgG) were used as negative control samples. Chondrocyte nuclei were stained with 4′,6-diamidino-2-phenylindol.[n] Sections were mounted with an antifade reagent[d] and examined with a light and fluorescence microscope, and digital images were captured.[r]

Statistical analysis—Least square mean data were analyzed by means of 2-way ANOVA with the variables time (1, 2, 12, or 48 hours), stimulation (unstimulated, IL-1β, or IL-6) and the interaction between time and stimulation included in the model. Time was considered as a categorical variable to determine the stimulation time that would be important for changes in GDF-5 mRNA expression. Significant variables identified in the 2-way ANOVA were analyzed with a multiple-comparisons procedure (Holm-Sidak method) to further identify the groups with significant differences within the variable. To evaluate the effect of cytokine stimulation, logarithmic gene expression values for 1- to 2-hour (short-term) and 12- to 48-hour (long-term) stimulations were compared with values for unstimulated chondrocytes with a pairwise *t* test. All statistical analyses were performed with software.[s] Values of $P < 0.05$ were considered significant.

Results

Gene expression in chondrocyte pellets—Results indicated a significant ($P = 0.002$) effect of stimulation on GDF-5 mRNA gene expression after controlling for the effects of time. No significant difference of time ($P = 0.059$) or interaction of time and stimulation ($P = 0.643$) on GDF-5 mRNA gene expression values was detected.

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

For the variable stimulation, significantly (P = 0.041) lower expression of GDF-5 was detected in pellets exposed to IL-1β versus unstimulated pellets. Additionally, a significant (P = 0.002) difference was detected in mean GDF-5 expression between chondrocyte pellets stimulated with IL-1β and those stimulated with IL-6; expression was higher for IL-6–stimulated pellets.

Results of analysis of the effect of cytokine stimulation at each time indicated significantly (P = 0.007) higher GDF-5 mRNA expression in chondrocyte pellets stimulated with IL-6 for 2 hours versus unstimulated pellets at that time (**Figure 1**). The mRNA expression of GDF-5 was significantly (P = 0.011) higher in chondrocyte pellets stimulated with IL-6 for 48 hours than it was for pellets stimulated with IL-1β for that length of time.

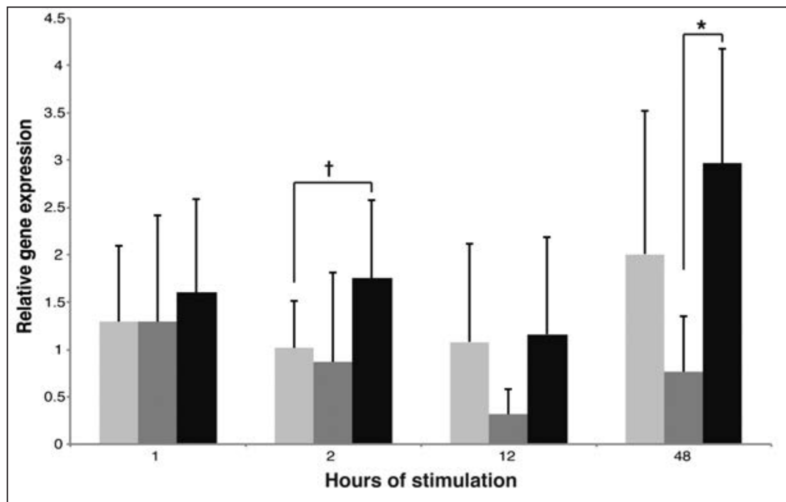

Figure 1—Mean ± SD relative GDF-5 mRNA gene expression in unstimulated control chondrocyte pellets (light gray bars) and pellets stimulated with IL-1β (dark gray bars) and IL-6 (black bars) for 1, 2, 12, and 48 hours (n = 4, 4, 3, and 4 horses, respectively). *Values are significantly ($P < 0.05$) different between the 2 groups indicated by the bracket. †Values are significantly ($P < 0.01$) different between the 2 groups indicated by the bracket.

Microarray global transcriptional analysis—Novel putative target genes were identified by screening to detect those with a low threshold of mean fold change in expression (> 1.5) that were likely to be affected (directly or indirectly) by stimulation with IL-6. After removing nonannotated transcripts, 59 (for 1 hour of stimulation) and 21 (for 2 hours of stimulation) genes regulated by IL-6 remained. Expression of CCDC88C, a negative regulator of the canonical Wnt signaling pathway,[23] was induced after 1 hour of IL-6 stimulation; on the basis of

Table 1—Genes of the canonical Wnt signaling pathway with differences in expression between chondrocyte pellets stimulated with IL-6 for 1 or 2 hours and unstimulated control pellets (n = 4 horses).

Gene name	Gene symbol	Fold difference in expression (1 hour)	Fold difference in expression (2 hours)
Extracellular			
Dickkopf homolog 1	*DKK1*	–1.10	—
Secreted frizzled-related protein 2	*SFRP2*	—	–1.10
WNT, member 1	*WNT1*	—	–1.10
WNT, member 10B	*WNT10B*	—	1.10
WNT, member 3	*WNT3*	1.10	—
WNT, member 8A	*WNT8A*	1.10	—
WNT, member 8B	*WNT8B*	–1.10	–1.10
WNT, member 9B	*WNT9B*	–1.10	—
WNT inhibitory factor 1	*WIF1*	1.10	1.10
Membrane bound			
FZD3	*FZD3*	—	–1.10
FZD8	*FZD8*	–1.10	—
Intracellular			
Beta-transducin repeat containing	*BTRC*	1.10	—
Calcyclin binding protein	*CACYBP*	—	1.10
Casein kinase 2, alpha prime polypeptide	*CSNK2A2*	1.10	—
β-catenin (catenin [cadherin-associated protein], beta 1, 88kDa)	*CTNNB1*	—	–1.04
CCDC88C	*CCDC88C*	2.10	—
F-box and WD repeat domain containing 11	*FBXW11*	–1.10	—
GSK 3 beta	*GSK3B*	1.13	—
PPARD	*PPARD*	1.10	—
Presenilin 1	*PSEN1*	1.10	—
Protein phosphatase 2, regulatory subunit B′, alpha	*PPP2R5A*	1.10	—
SMAD family member 4	*SMAD4*	1.10	—
SUMO1/sentrin/SMT3 specific peptidase 2	*SENP2*	1.10	—

Data indicate fold differences in gene expression between IL-6–stimulated chondrocyte pellets and unstimulated chondrocyte pellets determined with microarray global transcriptional analysis. Genes with differences in expression were identified on the basis of a mean fold difference in expression value of > 1.1 except for β–catenin, for which a specific search was performed. Genes with a positive fold difference value were upregulated in IL-6–stimulated chondrocyte pellets compared with expression in unstimulated control pellets. Genes with a negative fold difference value were downregulated in IL-6–stimulated chondrocyte pellets compared with expression in unstimulated control pellets.

— = Gene expression not different between IL-6–stimulated and unstimulated chondrocyte pellets.

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

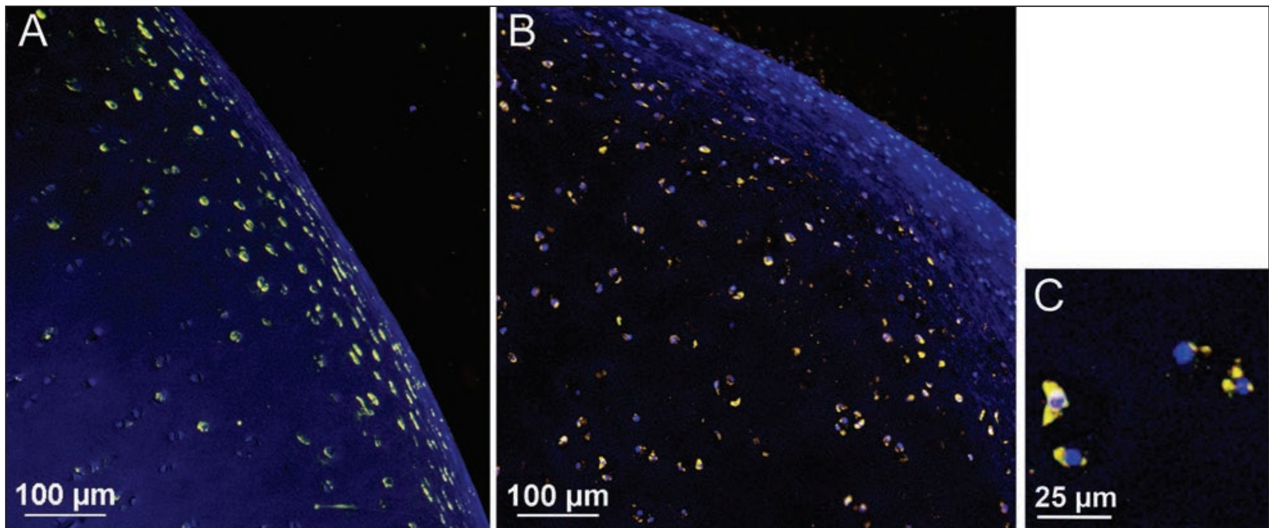

Figure 2—Representative photomicrographs of GDF-5 immunohistochemical staining in a sample of macroscopically normal cartilage obtained from the third carpal bone of a 2-year-old horse (A) and an OCF of that bone in a 10-year-old horse (B and C). Nuclei of chondrocytes are stained blue with 4′,6-diamidino-2-phenylindole. A—Notice that chondrocytes in the superficial and middle zones have GDF-5 immunostaining. B—Notice the GDF-5 immunostaining of chondrocytes in the middle and deep parts of the cartilage. C—Notice the distinct GDF-5 immunostaining in the cytoplasm and pericellular area.

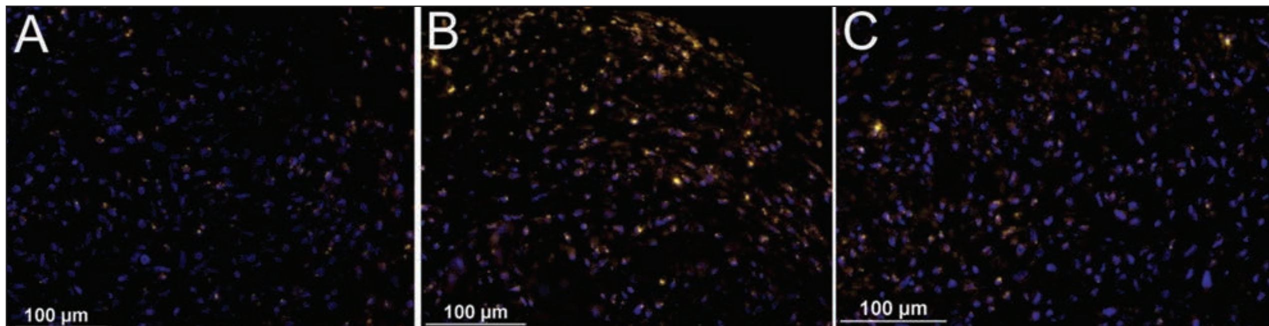

Figure 3—Representative photomicrographs of β-catenin staining in an unstimulated control chondrocyte pellet (A), a pellet stimulated with IL-1β for 1 hour (B), and a pellet stimulated with IL-6 for 1 hour (C). Nuclei were stained blue with 4′,6-diamidino-2-phenylindole. A—Notice that there are few stained cells. B—Notice the immunostaining in the center and in the periphery of the chondrocyte pellet. C— Notice that there are few stained cells.

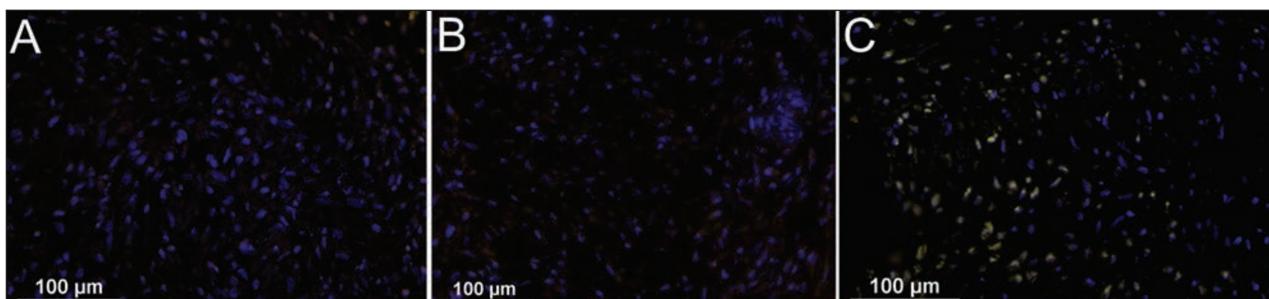

Figure 4—Representative photomicrographs of GSK-3β staining in an unstimulated chondrocyte control pellet (A), a pellet stimulated with IL-1β for 1 hour (B), and a pellet stimulated with IL-6 for 1 hour (C). Nuclei were stained blue with 4′,6-diamidino-2-phenylindole. A—Notice that there are no cells with GSK-3β immunostaining. B—Notice that there are few cells with GSK-3β immunostaining. C—Notice that there are many cells with GSK-3β immunostaining.

this finding, expression of other genes in the Wnt signaling pathway was evaluated. To minimize the risk of filtering out potentially relevant genes[24] in the pathway, a mean fold change in expression value of > 1.1 was used. A specific search for β-catenin was performed and results indicated a mean fold change of –1.04. Results indicated differences in expression of 23 canonical Wnt signaling pathway–related genes in IL-6–stimulated chondrocyte pellets (for 1- and 2-hour stimulation times), compared with expression in unstimulated pellets (**Table 1**).

Immunohistochemical analysis of GDF-5—Macroscopically normal articular cartilage samples had prominent cellular and pericellular staining of GDF-5 in the superficial and upper third of the middle zones[25] of the articular cartilage (**Figure 2**). However, in OCF samples, GDF-5 expression was localized in chondrocytes

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

and pericellular matrix in the middle and deep parts of the articular cartilage.

β-Catenin and GSK-3β expression in chondrocyte pellets stimulated with IL-6 for 1 hour—Prominent nuclear dephosphorylated β-catenin staining was detected in the center and periphery of chondrocyte pellets stimulated with IL-1β, but only a few cells had staining for β-catenin in pellets stimulated with IL-6; that finding was similar to findings for unstimulated chondrocyte pellets (**Figure 3**). Cellular immunostaining for GSK-3β was detected in a diffuse pattern in IL-6–stimulated chondrocyte pellets; only a few cells had GSK-3β staining in IL-1β–stimulated pellets, which was similar to findings for unstimulated pellets (**Figure 4**).

Discussion

The proinflammatory cytokine IL-1β has strong catabolic effects, and IL-6 is proposed to have a modulatory role in cartilage metabolism.[8,9] In the present study, the potential influences of IL-1β and IL-6 on expression of GDF-5 in chondrocytes in vitro was investigated. Both of these cytokines are considered to be important in the pathogenesis of OA.[8,9] Results of the present study indicated significant upregulation of GDF-5 expression within 2 hours after IL-6 stimulation. Significant downregulation of GDF-5 gene expression was found in all IL-1β stimulated pellets, compared with unstimulated pellets. That finding was in agreement with results of another in vitro study[26] that GDF-5 is strongly downregulated in human chondrocytes and fibroblast-like cells obtained from patients with rheumatoid arthritis grown in monolayer after 3, 6, and 18 hours of stimulation with IL-1β.

Results of the present study indicated regulation of GDF-5 gene expression by IL-6 and IL-1β was significantly different. Expression was lower for chondrocyte pellets stimulated with IL-1β than it was for pellets stimulated with IL-6. This result was in agreement with findings of another study[11] conducted in our laboratory that IL-1β downregulates sex determining region Y box 9 expression and IL-6 upregulates expression of that gene (although this result was not significant). These findings suggest that IL-1β has antichondrogenic effects and IL-6 has prochondrogenic effects.

Effects of cytokines on gene expression can be detected after a short time.[27] To determine the short-term effects of cytokine stimulation and identify potential mechanisms and signaling pathways involved, global transcriptional analysis with a microarray was performed for chondrocyte pellets that were stimulated for 1 and 2 hours in the present study; Wnt signaling–related genes were identified during that analysis. The Wnt signaling pathway has central roles in various developmental processes and regulates tissue induction and polarity. In the present study, the expressions of several inhibitors of the canonical Wnt signaling pathway were induced after 1 hour of stimulation with IL-6 (the extracellular Wnt inhibitory factor 1[19] and the intracellular negative regulators CCDC88C[28] and GSK-3β). Furthermore, decreased expression of the receptor FZD8 and the ligands WNT8B and WNT9B (ie, products of the genes *WNT8B* and *WNT9B*) and increased expression of negative regulators involved in the ubiquitin-mediated degradation of β-catenin (eg, β-transducin repeat containing gene; presenilin-1; sentrin-specific peptidase 2; and protein phosphatase 2, regulatory subunit B′, α[29–33]) were detected at that same time. This finding suggested that IL-6 stimulation inhibited the canonical Wnt signaling pathway. On the contrary, the ligands WNT3 and WNT8A and a positive regulator (casein kinase 2, α prime polypeptide[34]) were induced after 1 hour of IL-6 stimulation, whereas expression of an extracellular inhibitor, dickkopf homolog 1,[19] and an intracellular inhibitor, F-box and WD repeat domain containing 11[35] (which may both mediate degradation of β-catenin), were decreased at that same time, possibly indicating active Wnt signaling.

Results of the present study indicated upregulation of SMAD4 expression and downregulation of β-catenin expression after 1 and 2 hours of IL-6 stimulation, respectively. This finding supported results of another study[36] that increased SMAD4 expression enables BMP signaling to decrease β-catenin expression, resulting in inhibited Wnt signaling. In addition, results of a recent study[37] indicate BMP-2 induces activation of the Wnt–β-catenin pathway by means of increased LRP5 activity and that SMAD complexes bind to the LRP5 promoter with enhanced binding after BMP-2 treatment. Results of the present study are further evidence of the role of SMADs in the crosstalk between the Wnt and BMP signaling pathways. Results of microarray analysis in this study indicated increased expression of the canonical Wnt signaling pathway target gene PPARD[38] after 1 hour of stimulation with IL-6, which could have indicated an active canonical Wnt signaling pathway. However, PPARD is also involved in other mechanisms,[39] and because no additional target genes for the canonical Wnt signaling pathway were detected, we do not believe that increased expression of PPARD unambiguously indicated an active Wnt pathway. Inhibition of the canonical Wnt signaling pathway after 2 hours of cytokine stimulation was indicated by the finding of increased expression of Wnt inhibitory factor 1 and calcyclin binding protein[40] (extracellular and intracellular proteins, respectively) and a lack of detection of downstream target genes. Furthermore, expressions of the ligands WNT1 and WNT8B and the receptor FZD3, which potentially could activate the pathway, were downregulated at that same time. However, WNT10B expression was increased, and expression of the extracellular inhibitor secreted frizzled-related protein 2 was decreased. This finding could have indicated an active Wnt signaling pathway, although the possibility that the ligand interacted with other Wnt signaling pathways (other than the canonical pathway) could not be ruled out. The results of microarray analysis in this study indicated upregulation of several major canonical Wnt signaling pathway–related inhibitors and downregulation of the transcription factor β-catenin, suggesting that the canonical Wnt signaling pathway was inhibited by IL-6.

The transcriptional effects of IL-6 identified in this study were small in magnitude. In addition, a small number of microarray analyses were performed. There-

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

fore, statistical analysis was difficult. However, as indicated by results of another study,[24] transcriptional changes of a small magnitude are interesting findings when they occur for sets of related genes that participate in the same biological process, as for many Wnt-related genes.[41] On the contrary, CCDC88C had a larger change in gene expression than other Wnt-related genes in this study. This finding indicated that a high level of gene expression was needed to regulate and induce a substantial biological response in the canonical Wnt signaling pathway. However, further studies would be required to determine the importance of CCDC88C in such pathways.

Results of immunohistochemical analysis indicated stronger protein expression of nuclear dephosphorylated β-catenin in IL-1β–stimulated chondrocytes pellets after 1 hour, compared with expression in IL-6–stimulated and unstimulated chondrocyte pellets. Additionally, β-catenin accumulation was also suggested by the findings of a lack of GSK-3β staining in IL-1β–stimulated chondrocyte pellets and strong staining in IL-6–stimulated pellets. These findings may have been attributable to active Wnt signaling in chondrocyte pellets stimulated with IL-1β and a low level of Wnt signaling in pellets stimulated with IL-6, because nuclear translocation of β-catenin is a sign of activation of the Wnt–β-catenin signaling pathway.[42] Furthermore, IL-1β is associated with several Wnt-related markers[43–45] and may induce Wnt signaling by increasing expression of β-catenin.[46] Activation of the canonical Wnt signaling pathway induces OA cartilage features.[47,48] Therefore, findings of the present study may suggest that IL-6 could exert a protective effect on cartilage by inhibiting this pathway.

Contradictory roles of the canonical Wnt signaling pathway have been implicated in the regulation of GDF-5 expression.[18,49] Other authors[50] have suggested that SMAD and Wnt–β-catenin signals inhibit each other and are associated with maintenance of intervertebral disk homeostasis[50]; speculatively, that may also be true for articular cartilage. Such crosstalk between Wnt and BMP signaling pathways may explain the results of this study indicating the upregulation of GDF-5 may induce a downregulation of the Wnt pathway.

In the present study, chondrocytes of the superficial and upper third of the middle zones[25] of the macroscopically normal articular cartilage samples had positive immunostaining results for GDF-5. The superficial zone of macroscopically normal bovine, human,[51] and equine[52] articular cartilage contains progenitor cells. Results of the present study are in agreement with those of a study[13] of human articular cartilage collected after death that indicated discrete immunostaining for GDF-5 in the upper cartilage zones. However, results of another study[12] of human cartilage indicate GDF-5 is present in all cartilage zones. Mutations in the GDF-5 gene result in abnormal joint development and skeletal malformation diseases.[53] In addition, a single nucleotide polymorphism in GDF-5, rs143383, is associated with development of OA.[54] This suggests that tight regulation of GDF-5 expression is necessary not only during embryogenesis but also after birth.[53]

Chondrocytes in cartilage of OCFs obtained from middle carpal joints of horses with OA synthesize COMP[2] and express IL-6.[3] Results of the present study indicated chondrocytes and pericellular matrix in most zones of articular cartilage in OCFs had immunostaining for GDF-5. The finding that both IL-6 and GDF-5 were expressed in cartilage of OCFs indicated anabolic processes in such cartilage, possibly in an attempt to repair cartilage. That finding was consistent with findings of other studies that proteoglycan, collagen type II,[55] and COMP[2] synthesis are increased in joints with OCFs. In addition, dogs with experimentally induced OA have increased concentrations of IL-6 in synovial fluid, which correlates with increased proteglycan synthesis.[56] Results of another study[57] indicate IL-6 stimulation of human chondrocytes in vitro increases production of glycosaminoglycans. Further support for a chondrogenic role of IL-6 is indicated by results of other studies indicating human chondrocytes have increased gene expression of collagen type II, link protein, BMP-7, BMP receptors,[58] and tissue inhibitor of metallopeptidase-1[59] in the presence of IL-6 and the soluble IL-6 receptor.

Results of the present study indicated chondrocytes in macroscopically normal equine cartilage and cartilage obtained from joints of horses with OA express GDF-5. Upregulation of the GDF-5 gene was found after 2 hours of stimulation with IL-6; expression of that gene was highest after 48 hours of stimulation with IL-6, compared with results for chondrocyte pellets stimulated with IL-1β. Results of microarray analysis suggested that upregulation of GDF-5 expression may have been attributable to downregulation of the canonical Wnt signaling pathway induced by IL-6. This was supported by the finding of increased gene expression of several inhibitors of the canonical Wnt signaling cascade, loss of immunostaining for β-catenin, and increased staining for GSK-3β after short-term (1 hour) IL-6 stimulation. The findings of this study suggested that IL-6 may have a regulatory role via inhibition of the Wnt signaling pathway and subsequent upregulation of GDF-5 expression during repair of cartilage in joints of horses with OCFs. These findings may be of importance to understanding regenerative and growth mechanisms in cartilage and may be useful in the development of pharmacological treatments for enhancement of cartilage repair.

a. KT-400, Koneteolisuus Oy, Klaukkala, Finland.
b. R&D Systems, Abingdon, England.
c. Qiagen, Hilden, Germany.
d. Life Technologies, Paisley, Scotland.
e. National Center for Biotechnology Information. Available at www.ncbi.nlm.nih.gov. Accessed Aug 6, 2008.
f. ENSEMBLE, European Bioinformatics Institute and Wellcome Trust Sanger Institute, Hinxton, England. Available at: www.ensembl.org. Accessed Aug 6, 2008.
g. BLASTN National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md. Available at: blast.ncbi.nlm.nih.gov. Accessed Sep 6, 2009.
h. GenEx Enterprise 5.2.3.13, MultiD analyses, Gothenburg, Sweden.
i. Agilent 2100 Bioanalyzer, Agilent Technologies Inc, Santa Clara, Calif.
j. NanoDrop 1000, Thermo Scientific, Wilmington, Del.
k. Nottingham Arabidopsis Stock Centre, University of Nottingham, Nottingham, England.
l. Affymetrix Inc, Santa Clara, Calif.

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

m. NetAff Analysis Center. Available at: www.affymetrix.com/analysis/index.affx. Accessed Apr 4, 2012.
n. Sigma Aldrich, St Louis, Mo.
o. Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
p. Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.
q. TSA Cyanine 3 kit, NEL 704A, Perkin Elmer, Waltham, Mass.
r. NIS Elements imaging software, Nikon Instruments Inc, Tokyo, Japan.
s. SigmaPlot 12.5, Systat Software Inc, San Jose, Calif.

References

1. Pool RR, Meagher DM. Pathologic findings and pathogenesis of racetrack injuries. *Vet Clin North Am Equine Pract* 1990;6:1–30.
2. Skiöldebrand E, Heinegård D, Eloranta ML, et al. Enhanced concentration of COMP (cartilage oligomeric matrix protein) in osteochondral fractures from racing Thoroughbreds. *J Orthop Res* 2005;23:156–163.
3. Ley C, Ekman S, Elmen A, et al. Interleukin-6 and tumour necrosis factor in synovial fluid from horses with carpal joint pathology. *J Vet Med A Physiol Pathol Clin Med* 2007;54:346–351.
4. Ley C, Ekman S, Roneus B, et al. Interleukin-6 and high mobility group box protein-1 in synovial membranes and osteochondral fragments in equine osteoarthritis. *Res Vet Sci* 2009;86:490–497.
5. Heinegård D, Saxne T. The role of the cartilage matrix in osteoarthritis. *Nat Rev Rheumatol* 2011;7:50–56.
6. Guerne PA, Carson DA, Lotz M. IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. *J Immunol* 1990;144:499–505.
7. Lotz M, Terkeltaub R, Villiger PM. Cartilage and joint inflammation. Regulation of IL-8 expression by human articular chondrocytes. *J Immunol* 1992;148:466–473.
8. Goldring MB. The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. *Connect Tissue Res* 1999;40:1–11.
9. de Hooge AS, van de Loo FA, Bennink MB, et al. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. *Osteoarthritis Cartilage* 2005;13:66–73.
10. David F, Farley J, Huang H, et al. Cytokine and chemokine gene expression of IL-1beta stimulated equine articular chondrocytes. *Vet Surg* 2007;36:221–227.
11. Ley C, Svala E, Nilton A, et al. Effects of high mobility group box protein-1, interleukin-1beta, and interleukin-6 on cartilage matrix metabolism in three-dimensional equine chondrocyte cultures. *Connect Tissue Res* 2011;52:290–300.
12. Bobinac D, Spanjol J, Marinovic M, et al. Expression of bone morphogenetic proteins, cartilage-derived morphogenetic proteins and related receptors in normal and osteoarthritic human articular cartilage. *Coll Antropol* 2008;32(suppl 2):83–87.
13. Erlacher L, Ng CK, Ullrich R, et al. Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro. *Arthritis Rheum* 1998;41:263–273.
14. Francis-West PH, Abdelfattah A, Chen P, et al. Mechanisms of GDF-5 action during skeletal development. *Development* 1999;126:1305–1315.
15. Edwards CJ, Francis-West PH. Bone morphogenetic proteins in the development and healing of synovial joints. *Semin Arthritis Rheum* 2001;31:33–42.
16. Kwong FN, Hoyland JA, Evans CH, et al. Regional and cellular localisation of BMPs and their inhibitors' expression in human fractures. *Int Orthop* 2009;33:281–288.
17. Reddi AH. Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. *Ann Rheum Dis* 2003;62(suppl 2):ii73–ii78.
18. Guo X, Day TF, Jiang X, et al. Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev* 2004;18:2404–2417.
19. Baarsma HA, Konigshoff M, Gosens R. The Wnt signaling pathway from ligand secretion to gene transcription: molecular mechanisms and pharmacological targets. *Pharmacol Ther* 2013;138:66–83.
20. Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* 2008;4:68–75.
21. Nusse R. The WNT homepage. Available at: www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes. Accessed Jun 6, 2013.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408.
23. Oshita A, Kishida S, Kobayashi H, et al. Identification and characterization of a novel Dvl-binding protein that suppresses WNT signalling pathway. *Genes Cells* 2003;8:1005–1017.
24. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 2003;34:267–273.
25. Pritzker KP, Aigner T. Terminology of osteoarthritis cartilage and bone histopathology—a proposal for a consensus. *Osteoarthritis Cartilage* 2010;18(suppl 3):S7–S9.
26. Liu FL, Lin LH, Sytwu HK, et al. GDF-5 is suppressed by IL-1beta and enhances TGF-beta3-mediated chondrogenic differentiation in human rheumatoid fibroblast-like synoviocytes. *Exp Mol Pathol* 2010;88:163–170.
27. Vincenti MP, Brinckerhoff CE. Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1beta. *Arthritis Res* 2001;3:381–388.
28. Ekici AB, Hilfinger D, Jatzwauk M, et al. Disturbed Wnt signalling due to a mutation in CCDC88C causes an autosomal recessive non-syndromic hydrocephalus with medial diverticulum. *Mol Syndromol* 2010;1:99–112.
29. Kang DH, Fox C. Neuroendocrine and leukocyte responses and pulmonary function to acute stressors. *Ann Behav Med* 2000;22:276–285.
30. Kirschenbaum F, Hsu SC, Cordell B, et al. Substitution of a glycogen synthase kinase-3beta phosphorylation site in presenilin 1 separates presenilin function from beta-catenin signaling. *J Biol Chem* 2001;276:7366–7375.
31. Li X, Yost HJ, Virshup DM, et al. Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in *Xenopus*. *EMBO J* 2001;20:4122–4131.
32. Nishida T, Kaneko F, Kitagawa M, et al. Characterization of a novel mammalian SUMO-1/Smt3-specific isopeptidase, a homologue of rat axam, which is an axin-binding protein promoting beta-catenin degradation. *J Biol Chem* 2001;276:39060–39066.
33. Reifenberger J, Knobbe CB, Wolter M, et al. Molecular genetic analysis of malignant melanomas for aberrations of the Wnt signaling pathway genes CTNNB1, APC, ICAT and BTRC. *Int J Cancer* 2002;100:549–556.
34. Nguyen AV, Albers CG, Holcombe RF. Differentiation of tubular and villous adenomas based on Wnt pathway-related gene expression profiles. *Int J Mol Med* 2010;26:121–125.
35. Fuchs SY, Chen A, Xiong Y, et al. HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. *Oncogene* 1999;18:2039–2046.
36. Freeman TJ, Smith JJ, Chen X, et al. Smad4-mediated signaling inhibits intestinal neoplasia by inhibiting expression of beta-catenin. *Gastroenterology* 2012;142:562–571.
37. Papathanasiou I, Malizos KN, Tsezou A. Bone morphogenetic protein-2-induced WNT/beta-catenin signaling pathway activation through enhanced low-density-lipoprotein receptor-related protein 5 catabolic activity contributes to hypertrophy in osteoarthritic chondrocytes. *Arthritis Res Ther* 2012;14:R82.
38. Zandi PP, Belmonte PL, Willour VL, et al. Association study of Wnt signaling pathway genes in bipolar disorder. *Arch Gen Psychiatry* 2008;65:785–793.
39. van der Veen JN, Kruit JK, Havinga R, et al. Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1. *J Lipid Res* 2005;46:526–534.
40. Santelli E, Leone M, Li C, et al. Structural analysis of Siah1-Siah-interacting protein interactions and insights into the assembly of an E3 ligase multiprotein complex. *J Biol Chem* 2005;280:34278–34287.
41. Monroe DG, McGee-Lawrence ME, Oursler MJ, et al. Update on Wnt signaling in bone cell biology and bone disease. *Gene* 2012;492:1–18.

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC

42. Willert K, Nusse R. Beta-catenin: a key mediator of WNT signaling. *Curr Opin Genet Dev* 1998;8:95–102.
43. Ge X, Ma X, Meng J, et al. Role of WNT-5A in interleukin-1beta-induced matrix metalloproteinase expression in rabbit temporomandibular joint condylar chondrocytes. *Arthritis Rheum* 2009;60:2714–2722.
44. Thorfve A, Dehn T, Lindahl A, et al. Characteristic markers of the WNT signaling pathways are differentially expressed in osteoarthritic cartilage. *Cartilage* 2012;3:43–57.
45. Weng LH, Wang CJ, Ko JY, et al. Inflammation induction of Dickkopf-1 mediates chondrocyte apoptosis in osteoarthritic joint. *Osteoarthritis Cartilage* 2009;17:933–943.
46. Hwang SG, Ryu JH, Kim IC, et al. WNT-7a causes loss of differentiated phenotype and inhibits apoptosis of articular chondrocytes via different mechanisms. *J Biol Chem* 2004;279:26597–26604.
47. Corr M. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. *Nat Clin Pract Rheumatol* 2008;4:550–556.
48. Miclea RL, Siebelt M, Finos L, et al. Inhibition of Gsk3beta in cartilage induces osteoarthritic features through activation of the canonical WNT signaling pathway. *Osteoarthritis Cartilage* 2011;19:1363–1372.
49. Später D, Hill TP, Gruber M, et al. Role of canonical WNT-signalling in joint formation. *Eur Cell Mater* 2006;12:71–80.
50. Hiyama A, Sakai D, Tanaka M, et al. The relationship between the WNT/beta-catenin and TGF-beta/BMP signals in the intervertebral disc cell. *J Cell Physiol* 2011;226:1139–1148.
51. Dowthwaite GP, Bishop JC, Redman SN, et al. The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* 2004;117:889–897.
52. McCarthy HE, Bara JJ, Brakspear K, et al. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Vet J* 2012;192:345–351.
53. Hellman TV, Nickel J, Mueller TD. Missense mutations in GDF-5 signaling: molecular mechanisms behind skeletal malformation. In: Cooper D, ed. *Mutations in human genetic disease*. Rijeka, Croatia: InTech, 2012;11–54. Available at: www.intechopen.com/books/mutations-in-human-genetic-disease/missense-mutations-in-gdf-5-signaling-molecular-mechanisms-behind-skeletal-malformation. Accessed May 13, 2013.
54. Liu J, Cai W, Zhang H, et al. Rs143383 in the growth differentiation factor 5 (GDF5) gene significantly associated with osteoarthritis (OA)—a comprehensive meta-analysis. *Int J Med Sci* 2013;10:312–319.
55. Frisbie DD, Ray CS, Ionescu M, et al. Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. *Am J Vet Res* 1999;60:306–309.
56. Venn G, Nietfeld JJ, Duits AJ, et al. Elevated synovial fluid levels of interleukin-6 and tumor necrosis factor associated with early experimental canine osteoarthritis. *Arthritis Rheum* 1993;36:819–826.
57. Tsuchida AI, Beekhuizen M, Rutgers M, et al. Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model. *Arthritis Res Ther* 2012;14:R262.
58. Namba A, Aida Y, Suzuki N, et al. Effects of IL-6 and soluble IL-6 receptor on the expression of cartilage matrix proteins in human chondrocytes. *Connect Tissue Res* 2007;48:263–270.
59. Silacci P, Dayer JM, Desgeorges A, et al. Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1–induced collagenolytic activity. *J Biol Chem* 1998;273:13625–13629.

Unauthenticated | Downloaded 09/28/23 04:20 AM UTC