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Novel Small Leucine-Rich Protein Chondroadherin-like (CHADL) is Expressed in Cartilage and Modulates Chondrocyte Differentiation*

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*Running title: Tissue-specific Expression and Function of CHADL

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Background: Collagen-binding proteins regulate tissue-specific extracellular matrices.
Results: CHADL is enriched in cartilage, binds collagen, and modulates chondrocyte phenotype.
Conclusion: CHADL regulates chondrocyte microenvironment.
Significance: Characterizing novel collagen-associated proteins is crucial to understand the constitution and function of specialized extracellular matrices.

ABSTRACT

        The constitution and biophysical properties of extracellular matrices can dramatically influence cellular phenotype during development, homeostasis, or pathogenesis. These effects can be signaled through a differentially regulated assembly of collagen fibrils, orchestrated by a family of collagen-associated Small Leucine-Rich Proteins, SLRPs. In this report, we describe the tissue-specific expression and function of a previously uncharacterized SLRP – Chondroadherin-like (CHADL). We have developed antibodies against CHADL and, by immunohistochemistry, detected CHADL expression mainly in skeletal tissues, particularly in fetal cartilage and in pericellular space of adult chondrocytes. In situ hybridizations and immunoblots on tissue lysates confirmed this tissue-specific expression pattern. Recombinant CHADL bound collagen in cell culture, and inhibited *in vitro* collagen fibrillogenesis. After *Chadl* shRNA knockdown chondrogenic ATDC5 cells increased their differentiation, indicated by increased transcript levels of *Sox9, Ihh, Col2a1,* and *Col10a1*. The knockdown increased collagen II and aggrecan deposition in the cell layers. Microarray analysis of the knockdown samples suggested collagen receptor-related changes, although other upstream effects could not be excluded. Together, our data indicate that the novel SLRP CHADL is expressed in cartilaginous tissues, influences collagen fibrillogenesis, and modulates chondrocyte differentiation. CHADL appears to have a negative regulatory role, possibly ensuring the formation of a stable extracellular matrix.

        To gain a comprehensive understanding of connective tissue biology it is crucial to study proteins that regulate tissue- and function-specific collagen fibril assembly. Over the past three decades, several homologous Small Leucine-Rich Proteins (SLRPs[1]) have been identified and functionally evaluated; many of these proteins are extracellularly associated with collagen and can influence collagen fibrillogenesis in vitro (1) – for example, decorin, biglycan, fibromodulin, and

lumican. SLRP knockout mouse phenotypes reveal that the lack of a given SLRP cannot be compensated by another SLRP - collagen fibrils in specific knockout mouse tissues appear to assemble in disordered manner. This leads to tissue-specific phenotypes: decorin-deficient mice have fragile skin (2), lumican-deficient mice have opaque cornea (3), biglycan-deficient mice have osteoporotic bones (4), and fibromodulin-deficient mice have mechanically weak tendons with increased collagen cross-linking (5,6). Compound SLRP deficiency further aggravates the abnormal collagen fibril phenotype, suggesting concerted action of SLRPs during collagen fibrillogenesis (5,7,8). Therefore, the tissue-specific, or even temporo-specific, expression of SLRPs modulates the architecture and cross-linking of the growing collagen fibres. Some SLRPs can even inhibit each other's binding to collagen – e.g. fibromodulin and lumican (9-11), or asporin and decorin (12,13) – which contributes to another level of collagen fibrillogenesis regulation.

Not all SLRPs have been characterized – one yet undescribed is chondroadherin-like (CHADL). *CHADL* resides on chromosome 22, and is 19% homologous with chondroadherin. Chondroadherin is a collagen- and integrin α2β1-binding SLRP, expressed in cartilage and bone (14-17), whose deficiency in mouse leads to thinner cortical bone and longer proliferative growth plate zone (18). The conspicuous difference between CHADL and other SLRPs is its size – twice as large as most SLRPs, *CHADL* gene appears to have arisen by tandem duplication of an entire single SLRP gene, the middle gap having been joined by a proline- and arginine-rich linker domain. Also, the integrin-binding site of chondroadherin is not well-conserved in CHADL and, unlike other SLRPs, CHADL features several interspersed cysteine residues besides the conserved SLRP-characteristic cysteine loops in N- and C-terminal flanking (LRRNT, LRRCT) domains (Fig. 1A).

Here, we evaluated the expression profile and function of CHADL to assess the role of CHADL in connective tissue biology. We have detected CHADL primarily in extracellular matrices of cartilage tissues, found it to be associated with collagen and influence chondrocyte differentiation *in vitro*.

EXPERIMENTAL PROCEDURES
Reagents – Insulin, transferrin, sodium selenite, glucose oxidase, catalase, hygromycin was from Sigma. Nuclease-free water, PBS, SSC, Tris, Lipofectamine were from Life Technologies. αMEM, DMEM/F12, 293 expression media were Thermo Scientific. ATDC5 and HFL1 cell lines were from Sigma. *Chadl* Stellaris probes were from Biosearch Technologies. Ni-NTA affinity purification cartridge was from Qiagen. Antibodies were from Abcam (anti-collagen I (ab34710), anti-collagen III (ab7778), Pierce (anti-aggrecan PA1-1745), Genscript (anti-actin), or in-house (anti-collagen II).

Antibody against CHADL – Rabbit polyclonal antibody was made against the peptide FPSDTQLLDLRRNH, covering amino acids 423-436 of the human CHADL protein. (Corresponding mouse Chadl sequence is FPNDTQLLDLRRNH.) The antiserum was purified on Protein A sepharose, and the specificity was confirmed by immunoblotting against cell medium containing recombinant CHADL or against medium from non-transfected control cells.

Immunohistochemistry – Sections of frozen mouse embryos, ranging from E10-E17, and two-month-old mouse knee joints were fixed in 4% formalin in PBS for 5 min, rinsed in TBS, and incubated in 0.3% hydrogen peroxide for 15 min. The slides were then incubated with hyaluronidase and chondroitinase ABC for 15 min. After rinsing with TBS, the slides were blocked with 10% goat serum in TBS for 1 h, then incubated with anti-CHADL diluted to 1 ug/mL in TBS with 1% goat serum. The slides were then washed with TBS, and stained with Ultra-Sensitive ABC Rabbit

IgG Staining Kit (Pierce) and DAB Peroxidase Substrate Kit (Vectorlabs).

In situ hybridization – Frozen sections of mouse embryos or two-week old mouse articular cartilage were fixed with phosphate-buffered 4% formaldehyde for 10 min, washed twice with PBS, and permeabilized for 5 hours in 70% ethanol. The slides were equilibrated in wash buffer (2xSSC, 10% formamide) twice for 3 minutes, and hybridized overnight at 37 ˚C with 1 uM Stellaris *Chadl* anti-sense probes diluted in hybridization buffer (10% dextran sulfate, 2xSSC, 10% formamide). Slides were then incubated in wash buffer for 30 min at 37 ˚C, and once again in the same conditions but with 5 ng/mL DAPI. The slides were then resuspended in 2xSSC, equilibrated with anti-fade GLOX buffer (2xSSC, 0.4% glucose, 10 mM Tris-HCl pH 8.0), and incubated with GLOX buffer including glucose oxidase (1:100 v/v) and catalase (1:100 v/v). The slides were imaged in a fluorescence microscope.

Cell culture - ATDC5 cells were cultured in DMEM/F12 1:1 medium with 5% fetal bovine serum (HyClone), 30 nM sodium selenite, and 10 ug/mL transferrin, in 5% CO_2 atmosphere. For differentiation, 10 ug/mL insulin and 35 ug/mL ascorbate was added. HFL1 cells were cultured in αMEM medium with 10% fetal bovine serum and 50 ug/mL ascorbate.

Immunoblotting - PVDF membranes with human tissue lysates were purchased from Zyagen. Mouse cartilage andother connective tissue lysates were obtained by boiling the tissues in 1% SDS. Cell layer homogenates were made by solubilizing the cells in TRIzol reagent (Life Technologies) and purifying proteins according to manufacturer's instructions. Cell medium was mixed with SDS-PAGE loading buffer. Protein amounts were quantified using Micro-BCA assays (Pierce). 30 μg total protein was run on 4-12% Bis-Tris SDS-PAGE reducing gels (Genscript). Proteins were transferred to a nitrocellulose membrane. The membranes were immunoblotted using 5% milk in TBS for blocking, TBS with 0.5% Tween-20 for washing, and washing buffer with 0.5% milk as antibody diluent. All antibodies were used at 1 μg/mL and secondary HRP-conjugated donkey anti-rabbit or anti-mouse (Dako) were diluted 1:15,000. Blots were developed with SuperSignal West Dura (Pierce) and imaged using CCD camera.

Recombinant protein expression – cDNA for human CHADL was synthesized and cloned into pCEP4 vector, with 6x his-tag-encoding sequences added to both flanks. This construct was transfected into 293T cells using Lipofectamine 2000, and CHADL-expressing cells were cloned and selected using 250 ug/mL hygromycin. After expansion, cells were grown in 293 expression medium supplemented with 50 ug/mL hygromycin. Protein was purified from the collected medium using Ni-NTA cartridges coupled to Äkta chromatograph. The protein identity was confirmed using mass spectrometry.

Proximity ligation assay – the assay reagents Duolink® were purchased from Sigma. HFL1 cells were seeded on coverslips at 10,000 cells per well, and transfected with CHADL-pCEP construct using Lipofectamine 2000. For negative control, cells were transfected with a his-tagged, non-collagen-binding domain of fibromodulin (LRR domains 1-3) in pCEP vector. (This fibromodulin domain is partially homologous with CHADL LRR domains, but doesn't bind collagen, as shown previously (10).) After one day, the cells were briefly fixed with paraformaldehyde and processed for proximity ligation assay, according to the manufacturer's instructions. Interactions were detected using antibodies against his-tag (Abcam ab18184) and against procollagen (LF42, kind gift from Dr. Larry Fisher).

Collagen fibrillogenesis functional assay – the assay is described here (19). Briefly, 100 μg/mL of acid-extracted pepsinated collagen type I was neutralized into HEPES buffer with 0.15 M NaCl, and incubated with or without recombinant CHADL protein at 5 μg/mL. This ratio amounts to five-fold molar excess of

collagen. Samples were incubated at 37 ˚C in a spectrometer that continuously measured absorbance at 400 nm.

Co-sedimentation assay – 100 μg/mL collagen type I and 5 μg/mL CHADL (i.e. five-fold molar excess of collagen) were incubated in PBS buffer pH 7.4 at 37 ˚C for 8 h. The mixture was centrifuged at 20,000 x g, supernatant and pellet were separately mixed with SDS-PAGE loading buffer, run on a 4-12% Bis-Tris reducing gel, and transferred to a nitrocellulose membrane. Immunoblots for collagen and CHADL were performed using rabbit anti-collagen and rabbit anti-CHADL antibodies.

Cell-binding assay – the assay is described here (17). 48-well plate was pre-coated with CHADL, collagen II, or BSA, at 5 ug/mL in PBS. 105kc cells (chondrosarcoma) were seeded in PBS at 50,000 cells per well, incubated for 1 h, and the non-bound cells were washed off. The bound cells were quantified using lysosomal N-acetylglucosaminidase assay (20).

shRNA knockdown of Chadl expression in ATDC5 cells – 27-mer shRNA duplexes were purchased from Origene. The only effective knockdown was obtained from the construct ACAAGGUAGAGAAACAAAGAGACCA. Universal scrambled negative control duplex (Origene) was used as negative control. ATDC5 cells were transfected with 30 nM shRNA using Lipofectamine 2000, according to manufacturer's protocol. After 16 h incubation, the medium was changed to differentiation medium that was replaced every two days for the remaining course of the experiment. Knockdown of *Chadl* transcript was confirmed by qPCR using Taqman assays (Life Technologies) run on Applied Biosystems 7900 HT detection system. Cell cultures were used for microarray studies, and for assessing gene expression by qPCR and immunoblotting.

Alcian blue staining – Cells were rinsed with PBS three times and fixed with 95% ethanol for 5 min at -20 ˚C. Then, the cells were stained with 0.1% Alcian Blue 8GX in 0.1 M HCl overnight and washed three times with water. The staining was quantified by dissolving the dye in 6 M guanidine HCl overnight, and absorbance was read at 650 nm.

Microarrays – ATDC5 cells were transfected with *Chadl* shRNA and differentiated for five days. RNA was isolated using Quick-RNA kit (Zymo Research). aRNA was generated, hybridized onto an Affymetrix MG-430 PM array strip and processed according to the manufacturer's instruction. Two control samples and two different knockdown samples were used. The raw data was normalized using RMA (21) in which raw intensities are background-corrected, log2 transformed and then normalized using quantiles, as provided by R and Bioconductor (http://www.r-project.org). A linear model was fitted to the data using LIMMA (22) package to obtain expression value for each probe set. Probe sets with a log2 fold change > 0.5 and p value < 0.05 were considered differentially expressed. To functionally characterize the resulting gene lists, Database for Annotation, Visualization and Integrated Discovery (DAVID) (23) and Ingenuity Pathway Analysis (IPA) (Ingenuity Systems®) were employed. All microarray data were deposited into NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo) with accession number GSE57740.

RT-PCR and Real-Time PCR - Total RNA was extracted from cells with TRIzol reagent (Life Technologies). 100 ng RNA was used for reversed transcription using 1st Strand cDNA synthesis System (Origene). Real-time PCR was performed with TaqMan RNA-to-CT 1-Step Kit (Life Technologies) using Taqman probes, and Applied Biosystems 7900 HT detection system. Gene expression was normalized to *Actb* transcript.

RESULTS

Tissue expression profiling of Chadl – To test the specificity of our anti-CHADL antibody, we performed immunoblotting on medium collected from 293 cells transfected with CHADL expression vector. As a

negative control we used medium collected from non-transfected cells. Only the cells expressing CHADL produced protein detected as an 80 kDa band with the anti-CHADL antibody (Fig. 1B). Pre-immune antiserum did not recognize recombinant CHADL (data not shown).

To evaluate where Chadl is expressed in mouse, we performed immunohistochemistry on whole-mount mouse embryos, using the anti-CHADL antibody. We could not detect Chadl in whole-mount embryos before E13.5 (data not shown), at which stage, and in later developmental stages, the staining was prominent only in mesenchymal condensations and in cartilaginous tissues, and not present in any other tissues. Fig. 1 C,D shows a typical staining in chondrogenic condensations at E14.5. We also detected Chadl in two-month-old articular knee cartilage (Fig. 1E), where the protein was localized to extracellular space in the area immediately surrounding the chondrocytes (Fig. 1F), and no apparent staining appeared in other limb tissues (data not shown). This finding was in concert with immunoblotting of mouse cartilage extract with anti-CHADL - an 80 kDa Chadl band could be detected in articular cartilage (Fig. 1E inset). We also performed immunoblots of other tissue extracts but could not detect Chadl in any of the following organs: brain, stomach, intestine, colon, liver, lung, kidney, heart, ovary, skeletal muscle, spleen, testis, thymus, placenta, pancreas; we did not detect Chadl in other connective tissues, including skin, ligament, tendon, meniscus, bone (data not shown).

In situ hybridization using *Chadl* anti-sense probes on whole-mount mouse embryos also revealed high *Chadl* transcript level in the mesenchymal condensations and in cartilage, and we also detected *Chadl* transcripts in articular cartilage (Fig. 2).

Altogether, data from the different tissue profiling methods suggest that CHADL is expressed preferentially in embryonic developing skeleton and in extracellular matrix of adult cartilage.

Functional relevance of Chadl in collagen biology – One half of CHADL is 40% homologous with the collagen-binding protein chondroadherin. To investigate whether CHADL also associates with collagen we used proximity ligation assays on collagen-producing HFL1 cells transfected with his-tag-CHADL-pCEP expression vector. The assay revealed a strong association of CHADL with collagen, which was not observed in cells transfected with a non-collagen-binding his-tagged fibromodulin fragment (Fig. 3A).

We also investigated if recombinant CHADL (Fig. 3B) could influence collagen fibrillogenesis in vitro. While the control reaction progressed through a steady log phase, eventually reaching a plateau, supplementing the reaction with CHADL at a molar ratio collagen:CHADL 5:1 (five-fold molar excess of collagen) reduced the extent of fibrillogenesis by over 90% (Fig. 3C). In a similar experiment, we mixed collagen and CHADL at a five-fold molar excess of collagen, allowed fibrils to form, and then centrifuged the sample to spin down the fibrils. The pellet and the supernatant were separately analyzed by immunoblotting for collagen and CHADL. CHADL was present exclusively in the pelleted fraction of collagens (Fig. 3D).

Because the closely homologue chondroadherin interacts with chondrocytes via integrins, we also tested if CHADL can influence cell binding; however, we could not detect any interactions between chondrocytes and CHADL (Fig. 3E).

Effect of Chadl knockdown on differentiating chondrogenic cells – Because the tissue profiling data suggest specific function of Chadl in chondrocyte biology, we hypothesized that Chadl knockdown in a chondrogenic cell would influence specific cellular functions, pertinent to cartilage biology. Therefore, we studied the effects of *Chadl* knockdown in the chondrogenic ATDC5 cells.

To begin with, we examined the temporal *Chadl* expression pattern in the differentiating ATDC5 cells using both qPCR and immunoblotting. Chadl

transcripts increased in early stages of differentiation and persisted until day 20, after which they decreased (Fig. 4A). Evaluated by immunoblotting, Chadl protein levels varied only slightly during the entire differentiation period. Notably, Chadl appears to be absent in the pre-chondrogenic, non-differentating, stage (Fig. 4B).

Next, we analyzed the effects of *Chadl* shRNA-mediated knockdown on the differentiating ATDC5 cells. The shRNA construct induced over 75% *Chadl* knockdown between days 3 and 7 post-transfection after which the *Chadl* levels started to increase (evaluated by qPCR; data not shown). We analyzed if the knockdown of *Chadl* during early differentiation induced long-term effects on the cartilaginous phenotype of ATDC5 cells. In general, *Chadl* knockdown enhanced the differentiation of the cells. More intense Alcian blue staining indicated increased cartilage matrix proteoglycan production in cells treated with *Chadl* shRNA (Fig. 4C); although not statistically significant, data from three independent experiments showed a similar trend. More dramatic changes were observed by qPCR: at day 14 post-transfection, the knockdown samples had markedly increased levels of *Sox9* and *Ihh,* both associated with increased chondrocyte differentiation. We also detected a general increase of transcripts of many matrix-related genes, including collagens and some SLRPs (Fig. 4D), along with increased deposition of collagen II and aggrecan in the cell layers, but with minor or no changes in the amount of collagens I and III (Fig. 4E). (We did not observe any non-specific interferon response or cell stress response induced by the shRNAs, as evaluated by mRNA microarray; we did not detect any changes in expression of interferon-response genes *Oas1, Oas2, Ifitm1, Mx1*, or of ER-stress associated PDI, calreticulin, or autophagy-associated LC3).

Effects of Chadl knockdown assessed by microarrays – We used microarrays to assess the signaling and transcriptional profiles following *Chadl*

knockdown. We analyzed transcripts from day 4 after shRNA transfection, when the knockdown effect was strongest. Hierarchical clustering of the top 300 genes by variance showed that *Chadl* knockdown resulted in a contrasting transcriptional response (data are deposited in NCBI's Gene Expression Omnibus (24) accessible through GEO Series accession number GSE57740). Using differential expression analysis with a log2 fold change cut-off of 1.0, 56 non-redundant genes were upregulated whereas 46 genes were downregulated. With a lower cut-off of 0.5, 293 upregulated and 199 downregulated genes were detected. Diseases and functional categories predicted to be activated were related to growth and proliferation (z-score>2.0), with the transcriptome profiles being common in angiogenesis, growth of neuritis, proliferation of connective tissue cells, and formation of cellular protrusions (Fig. 5A). The relevant genes were the upregulated insulin-like growth factor binding protein 3 (IGFBP3), vascular cell adhesion molecule 1 (VCAM1), and MET proto-oncogene, and the downregulated histone deacetylase 9 (Hdac9) and cyclin-dependent kinase inhibitor 1A (CDKN1A) (Fig. 5B). Utilizing clustering function of DAVID (based on Gene Ontology terms) the top categories were consistent and comprised positive regulation of developmental process, and cell differentiation and proliferation. Genes associated with the extracellular structure organization, ossification and skeletal system development were also enriched.

Using Upstream Regulator Analysis to identify upstream regulator cascades, the predictions were consistent with a phenotype showing enhanced differentiation. RelA (p65) was predicted to be activated (activation z-score = 2.125), and is known to upregulate Sox9 (25) – master regulator of chondrogenesis. In addition, other differentiation regulators were predicted to be activated: NFKB, STAT1, STAT3 and p38 MAPK (activation z-scores = 3.652, 4.040, 4.272 and 2.414, respectively) (Fig. 5C). Thus, these findings are consistent with

the functional role of Chadl in chondrogenesis.

DISCUSSION

Extracellular matrices' influence on cellular behavior significantly depends on the structure of the collagenous matrix. This structure can be modulated by a family of collagen-binding SLRPs, whose temporo-spatially regulated expression contributes to the fine regulation of the growing collagen fibrils (1-3,5-7,11,13). To develop a comprehensive understanding of this process, it is necessary to identify and characterize the function of SLRPs. In this report we profiled the expression and evaluated the function of a previously undescribed SLRP – chondroadherin-like (CHADL). We observed CHADL to be expressed preferentially in embryonic cartilaginous tissues as well as in developing and in adult cartilage (Figs. 1,2). Interestingly, the expression in the embryonic tissues did not appear until E13.5, correlating with the onset of extracellular matrix production and chondrogenic differentiation. Using our antibodies and in situ hybridization probes, we could not detect CHADL in other major organs or other connective tissues; of course, we cannot exclude CHADL being expressed in other contexts, e.g. in specific pathological conditions, or being present in amounts below the detection limit of our methods. In cartilage, the protein is deposited in the extracellular matrix, in the pericellular matrix, but not in the interterritorial space. This could allude to its potential function – regulating early collagen fibrillogenesis in cartilage. Furthermore, the expression of CHADL appeared to persist in mesenchymal tissues and cartilage from E13.5 on to the adulthood, suggesting its role in maintaining homeostatis in the cartilage matrix. Altogether, this apparent tissue specificity could render CHADL useful as a biomarker for cartilage-related disorders, e.g. arthritis. The presence of CHADL in synovial fluid or blood samples and correlation with specific joint disorders deserves further attention.

What is the function of CHADL in cartilage? Since many SLRPs are collagen-associated, we investigated if CHADL also interacts with and influences collagen fibrillogenesis. Proximity ligation assays showed that CHADL indeed associates with collagen, and that recombinant CHADL, at low molarity, reduces in vitro collagen fibrillogenesis and binds to collagen fibrils (Fig. 3). Interestingly, chondroadherin, while generally sharing the tissue-specific expression pattern of CHADL and interacting with collagen, does not change the course of fibrillogenesis[2]. This can be due to the more bulky nature of CHADL – being twice the size of chondroadherin, the potential for steric hindrance of collagen fibril assembly is improved. CHADL could also contain other collagen-binding sites, targeted for a very effective restraining of the fibril assembly. Such function could be important for maintaining a proper collagen structure near the surface of a chondrocyte where collagen matrix is less dense than farther from the cell, in the interterritorial space.

In addition, CHADL does not share the integrin-mediated cell-binding properties of chondroadherin(16), making it functionally disparate from its close homologue (Fig. 3E). Indeed, for this and other unique features of CHADL mentioned earlier, the name Chondroadherin-like is misleading when considering its functional properties.

Because of its effect on collagen fibrillogenesis, CHADL could influence collagen receptor-mediated cellular response. After *Chadl* knockdown, microarray analysis indicated activation of STAT1/3 and p38 MAPK. STAT1/3 and p38 MAPK can be regulated by collagen-binding integrin α2β1 and DDR collagen receptor signaling (26,27). In addition, p65 – inducer of Sox9 in early chondrocyte differentiation (25) – was predicted to be activated (Fig. 5). The knockdown also accelerated longer term chondrocyte differentiation. Several days after the knockdown, chondrocyte differentiation markers *Col2a1*, *Sox9*, *Col10a1* and *Ihh*

were upregulated, along with the synthesis of collagen and proteoglycans (Fig. 4). These transcripts were unaffected at day 4, probably due to low basal expression at the early stage of differentiation. Taken together, chondrocyte differentiation was accelerated after *Chadl* knockdown, some of which may have been triggered by an altered collagen structure.

In summary, the novel SLRP chondroadherin-like (CHADL) is an extracellular matrix protein, expressed in developing cartilaginous tissues, and in young and adult cartilage. CHADL is sequestered to pericellular space of the chondrocytes, and associates with collagen, modulating collagen fibrillogenesis. This regulatory role in chondrocyte's collagenous microenvironment appears to influence cell differentiation, evidenced by our *Chadl* knockdown studies. Indeed, CHADL appears to have a negative regulatory role during chondrocyte differentiation, possibly ensuring the formation of a stable extracellular matrix. Further animal studies are required to determine the impact of CHADL in vivo, mainly to what extent CHADL is restricted to chondrogenesis of articular cartilage, cartilage maturation and degradation, or if CHADL can function during fracture healing or tendon/ligament injuries. It also remains to be investigated if CHADL can be a useful biomarker in specific joint and skeletal disorders.

REFERENCES

1. Kalamajski, S., and Oldberg, A. (2010) The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix biology : journal of the International Society for Matrix Biology* **29**, 248-253
2. Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* **136**, 729-743
3. Chakravarti, S., Petroll, W. M., Hassell, J. R., Jester, J. V., Lass, J. H., Paul, J., and Birk, D. E. (2000) Corneal opacity in lumican-null mice: defects in collagen fibril structure and packing in the posterior stroma. *Invest Ophthalmol Vis Sci* **41**, 3365-3373
4. Xu, T., Bianco, P., Fisher, L. W., Longenecker, G., Smith, E., Goldstein, S., Bonadio, J., Boskey, A., Heegaard, A. M., Sommer, B., Satomura, K., Dominguez, P., Zhao, C., Kulkarni, A. B., Robey, P. G., and Young, M. F. (1998) Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nat Genet* **20**, 78-82
5. Jepsen, K. J., Wu, F., Peragallo, J. H., Paul, J., Roberts, L., Ezura, Y., Oldberg, A., Birk, D. E., and Chakravarti, S. (2002) A syndrome of joint laxity and impaired tendon integrity in lumican- and fibromodulin-deficient mice. *J Biol Chem* **277**, 35532-35540
6. Kalamajski, S., Liu, C., Tillgren, V., Rubin, K., Oldberg, A., Rai, J., Weis, M., and Eyre, D. R. (2014) Increased C-Telopeptide Cross-linking of Tendon Type I Collagen in Fibromodulin-deficient Mice. *J Biol Chem*
7. Ezura, Y., Chakravarti, S., Oldberg, A., Chervoneva, I., and Birk, D. E. (2000) Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons. *J Cell Biol* **151**, 779-788
8. Zhang, G., Chen, S., Goldoni, S., Calder, B. W., Simpson, H. C., Owens, R. T., McQuillan, D. J., Young, M. F., Iozzo, R. V., and Birk, D. E. (2009) Genetic evidence for the coordinated regulation of collagen fibrillogenesis in the cornea by decorin and biglycan. *J Biol Chem* **284**, 8888-8897
9. Svensson, L., Narlid, I., and Oldberg, A. (2000) Fibromodulin and lumican bind to the same region on collagen type I fibrils. *FEBS letters* **470**, 178-182
10. Kalamajski, S., and Oldberg, A. (2007) Fibromodulin binds collagen type I via Glu-353 and Lys-355 in leucine-rich repeat 11. *J Biol Chem* **282**, 26740-26745

11. Kalamajski, S., and Oldberg, A. (2009) Homologous sequence in lumican and fibromodulin leucine-rich repeat 5-7 competes for collagen binding. *J Biol Chem* **284**, 534-539

12. Kalamajski, S., Aspberg, A., and Oldberg, A. (2007) The decorin sequence SYIRIADTNIT binds collagen type I. *J Biol Chem* **282**, 16062-16067

13. Kalamajski, S., Aspberg, A., Lindblom, K., Heinegard, D., and Oldberg, A. (2009) Asporin competes with decorin for collagen binding, binds calcium and promotes osteoblast collagen mineralization. *Biochem J* **423**, 53-59

14. Larsson, T., Sommarin, Y., Paulsson, M., Antonsson, P., Hedbom, E., Wendel, M., and Heinegard, D. (1991) Cartilage matrix proteins. A basic 36-kDa protein with a restricted distribution to cartilage and bone. *J Biol Chem* **266**, 20428-20433

15. Neame, P. J., Sommarin, Y., Boynton, R. E., and Heinegard, D. (1994) The structure of a 38-kDa leucine-rich protein (chondroadherin) isolated from bovine cartilage. *J Biol Chem* **269**, 21547-21554

16. Camper, L., Heinegard, D., and Lundgren-Akerlund, E. (1997) Integrin alpha2beta1 is a receptor for the cartilage matrix protein chondroadherin. *J Cell Biol* **138**, 1159-1167

17. Haglund, L., Tillgren, V., Addis, L., Wenglen, C., Recklies, A., and Heinegard, D. (2011) Identification and characterization of the integrin alpha2beta1 binding motif in chondroadherin mediating cell attachment. *J Biol Chem* **286**, 3925-3934

18. Hessle, L., Stordalen, G. A., Wenglen, C., Petzold, C., Tanner, E., Brorson, S. H., Baekkevold, E. S., Onnerfjord, P., Reinholt, F. P., and Heinegard, D. (2013) The skeletal phenotype of chondroadherin deficient mice. *PloS one* **8**, e63080

19. Hedbom, E., and Heinegard, D. (1989) Interaction of a 59-kDa connective tissue matrix protein with collagen I and collagen II. *J Biol Chem* **264**, 6898-6905

20. Landegren, U. (1984) Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. *Journal of immunological methods* **67**, 379-388

21. Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic acids research* **31**, e15

22. Wettenhall, J. M., and Smyth, G. K. (2004) limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics* **20**, 3705-3706

23. Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., and Lempicki, R. A. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome biology* **4**, P3

24. Edgar, R., Domrachev, M., and Lash, A. E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* **30**, 207-210

25. Caron, M. M., Emans, P. J., Surtel, D. A., Cremers, A., Voncken, J. W., Welting, T. J., and van Rhijn, L. W. (2012) Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. *PloS one* **7**, e33467

26. Xu, H., Bihan, D., Chang, F., Huang, P. H., Farndale, R. W., and Leitinger, B. (2012) Discoidin domain receptors promote alpha1beta1- and alpha2beta1-integrin mediated cell adhesion to collagen by enhancing integrin activation. *PloS one* **7**, e52209

27. Xu, L., Peng, H., Glasson, S., Lee, P. L., Hu, K., Ijiri, K., Olsen, B. R., Goldring, M. B., and Li, Y. (2007) Increased expression of the collagen receptor discoidin domain receptor 2 in articular cartilage as a key event in the pathogenesis of osteoarthritis. *Arthritis and rheumatism* **56**, 2663-2673

FOOTNOTES

[1]The abbreviations used are: SLRP, small leucine-rich repeat protein; LRR, leucine-rich repeat.
[2]V. Tillgren – unpublished observation.

FIGURE LEGENDS

FIGURE 1. A. Domain distribution in CHADL. Each half of CHADL contains several Leucine-rich domains (LRR) flanked by LRRNT and LRRCT domains. Both halves are linked by a proline- and arginine-rich domain. B. Characterization of anti-CHADL antibody. Immunoblots performed on medium collected from control, non-transfected, cells (lane 1), CHADL-expressing cells transfected with CHADL-pCEP4 expression vector (lane 2), and cell lysate from chondrogenic ATDC5 cells (lane 3). C. Whole-mount mouse E14.5 embryos stained with anti-CHADL (left) and isotype negative controls (right). Stainings were most prominent in mesenchymal condensations (arrows). Mesenchymal condensations in the marked areas are magnified in D. Two-month-old knee joint cartilage stained in E. Inset shows immunoblot of cartilage extract probed with anti-CHADL. F. shows magnified image of E. to demonstrate the pericellular distribution of CHADL.

FIGURE 2. In situ hybridization of *Chadl* transcripts in embryonic chondrogenic tissues and in two-week-old articular cartilage. Frozen tissue sections were briefly fixed, permeabilized with ethanol, and probed with a mix of anti-sense *Chadl* Stellaris red fluoresent probes. Sections are counter-stained with DAPI (blue nuclei).

FIGURE 3. Interaction of CHADL with collagen. A. Proximity ligation assay on HFL1 fibroblasts transfected with expression vector containing his-tagged CHADL. Cells were fixed, incubated with mouse anti-his and rabbit anti-procollagen antibodies, then processed for PLA using manufacturer's secondary antibodies and reagents. Protein interactions are detected by red fluorescence. Cells are counter-stained with DAPI (blue nuclei). On the right, negative control: cells were transfected with a non-collagen-binding, his-tagged, fibromodulin fragment containing LRR1-3 domains. . B. Coomassie-stained gel of Ni-NTA purified recombinant his-tagged CHADL expressed in human 293 cells. C. Collagen fibrillogenesis in vitro assay. Pepsin-extracted collagen was neutralized in HEPES-buffered saline buffer and supplemented with recombinant CHADL at a molar ratio 5:1. Control sample was without CHADL. Samples were incubated at 37 ˚C and absorbance at 400 nm was continuously recorded to follow fibrillogenesis in real time. D. Co-sedimentation assay. 3 μg collagen was incubated with or without 150 ng CHADL in PBS (molar ratio collagen:CHADL 5:1), incubated at 37 ˚C, and centrifuged after 8 h to separate the formed fibrils from soluble collagens. The two collagen fractions (pellet, *P*, and supernatant, *S*) were run on SDS-PAGE and immunoblotted for collagen and CHADL. E. Cell-binding assay. Chondrocytes were added to wells pre-coated with CHADL, collagen II (positive control), or BSA (negative control). After 1 hour the non-bound cells were removed, and the bound cells were quantified using lysosomal N-acetylglucosaminidase assay. Error bars show standard deviation (n=3).

FIGURE 4. A. Expression of *Chadl* in differentiating chondrogenic ATDC5 cells measured by qPCR using Taqman assays (n=3). Transcript levels are normalized to levels in pre-differentiation stage (day 0). B. Immunoblotting of differentiating ATDC5 cells. Cells were solubilized in

TRIzol at different differentiation days. Proteins were extracted and run on reducing SDS-PAGE, transferred to nitrocellulose membrane, and blotted for CHADL. C. Alcian blue staining and quantification of differentiating ATDC5 cells after *Chadl* shRNA knockdown or negative control shRNA. Cells were fixed at the indicated differentiation days and stained for proteoglycans using Alcian blue. Staining was quantified by measuring absorbance at 650 nm after solubilizing the stained samples (n=4) with guanidine. D. qPCR on differentiation markers (*Ihh, Col10a1, Sox9, Col2a1*), and extracellular matrix genes in Chadl shRNA knockdown-treated or control shRNA-treated differentiating ATDC5 cells (n=3) at differentiation day 14. Transcript levels were normalized to *Actb*.For clarity, deltaCt values and SD values are rounded off; percent change was calculated from deltadeltaCt (not shown) using original values. Asterisks mark changes with p<0.05 calculated using unpaired t-test. *Ctrl* is control and *KD* is knockdown sample. E. Immunoblots on collagens and aggrecan, using cell layer material extracted with TRIzol, or cell medium, from cultures used in D. Proteins were run on a 4-12% Bis-Tris reduced SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for collagens I, II, and III, aggrecan G3 domain (here, cleaved off the full-length aggrecan), and actin.

FIGURE 5. Transcriptomics analysis of *Chadl* shRNA knockdown in ATDC5 cells. A. Diseases and biological functions for the differentially expressed genes, with their respective activation p values, prediction states and activation z-scores. B. A network indicating the effects of the differentially regulated genes (outer circle) on individual diseases and functions categories (inner circle). C. Identification of regulator cascades upstream of the observed transcriptional changes by Upstream regulator analysis.
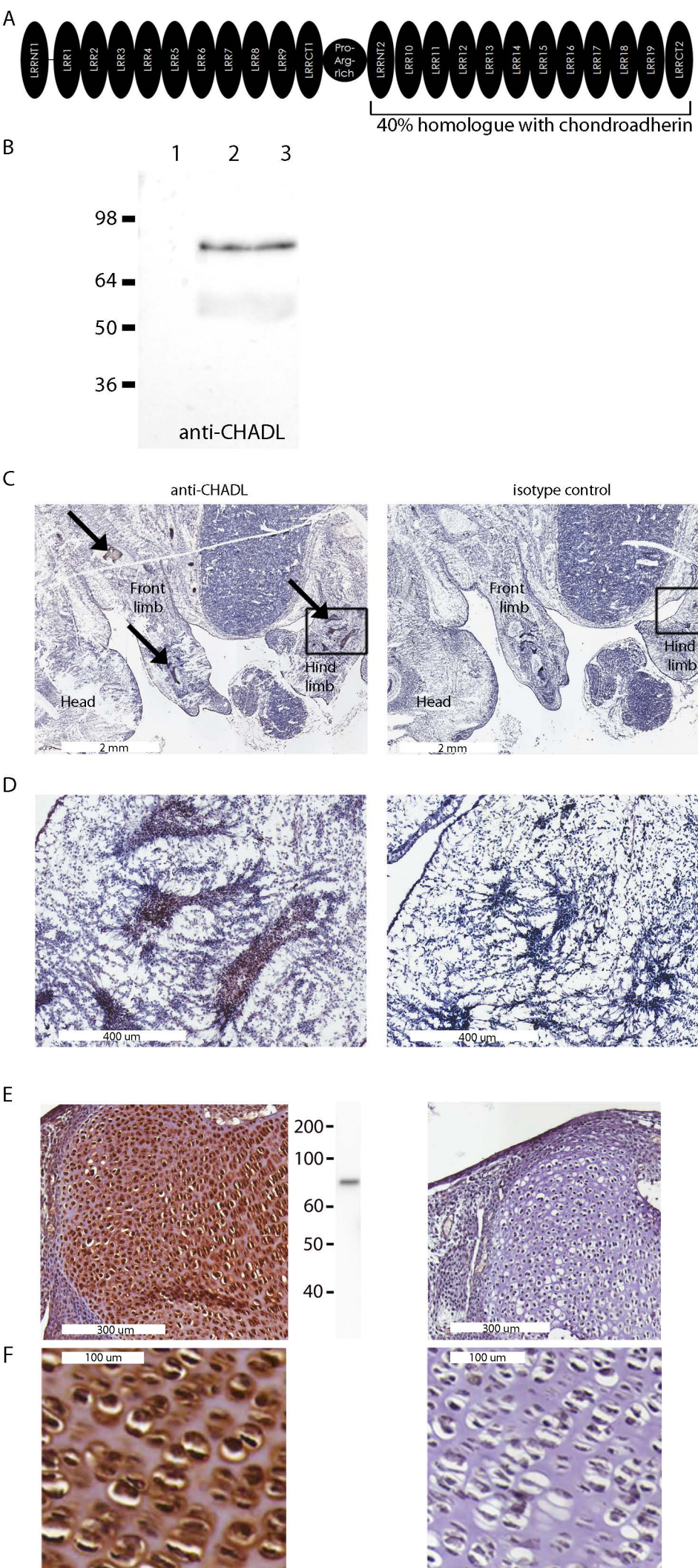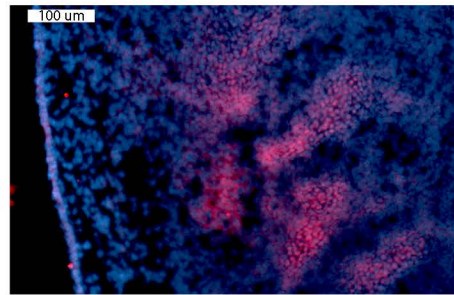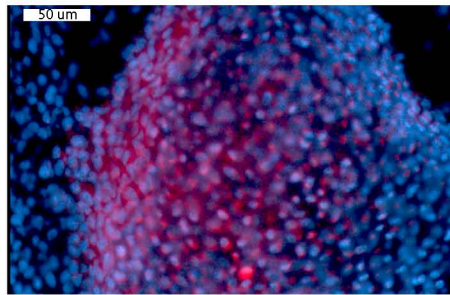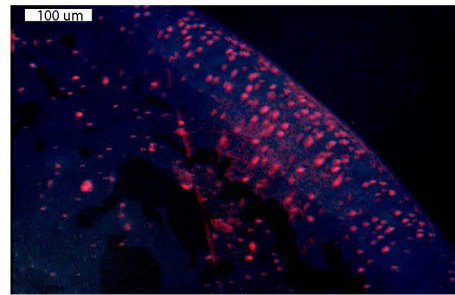
Figure 1

A

LRRNT1 | LRR1 | LRR2 | LRR3 | LRR4 | LRR5 | LRR6 | LRR7 | LRR8 | LRR9 | LRRCT1 | Pro-Arg-rich | LRRNT2 | LRR10 | LRR11 | LRR12 | LRR13 | LRR14 | LRR15 | LRR16 | LRR17 | LRR18 | LRR19 | LRRCT2

40% homologue with chondroadherin

B

1   2   3

98
64
50
36

anti-CHADL

C

anti-CHADL

Head   Front limb   Hind limb   2 mm

isotype control

Head   Front limb   Hind limb   2 mm

D

400 um   400 um

E

200
100
60
50
40

300 um   300 um

F

100 um   100 um

Figure 2
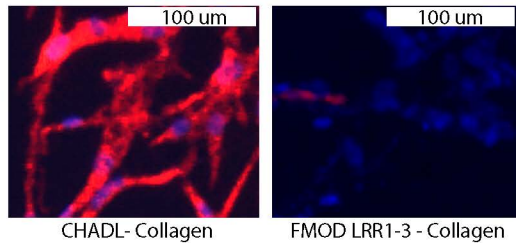

Chadl in E14.5 mesenchymal condensations

Chadl in E17.5 cartilage

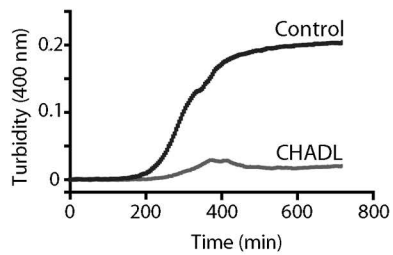*Chadl* in two-week-old articular cartilage

Figure 3

A

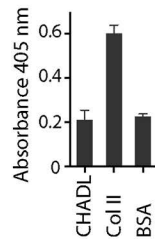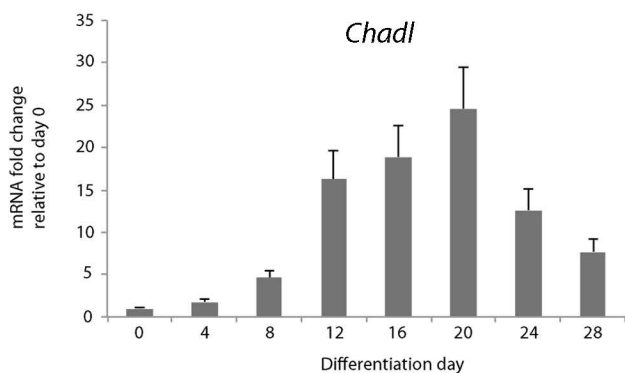CHADL- Collagen   FMOD LRR1-3 - Collagen

B

200 –
100 –
← CHADL
60 –
50 –
40 –

C

Turbidity (400 nm)

Control

CHADL

Time (min)

D

        S   P   S   P
                    200 –
                    100 –   ← Collagen
                     60 –   ← CHADL

w/o CHADL   with CHADL

E

Absorbance 405 nm

CHADL   Col II   BSA

Figure 4

A

B

Differentiation days:

0 4 7 10 14 21 28

98

64

50

36

anti-CHADL
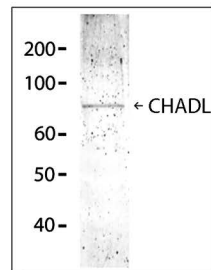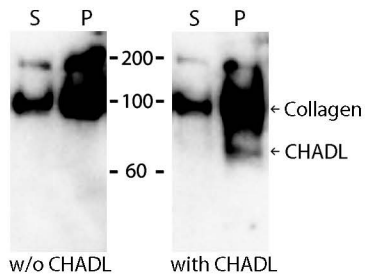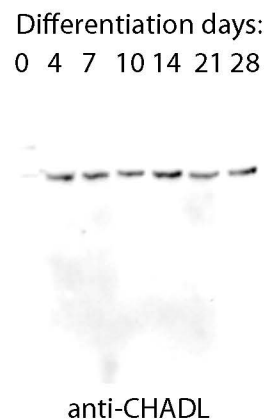
C
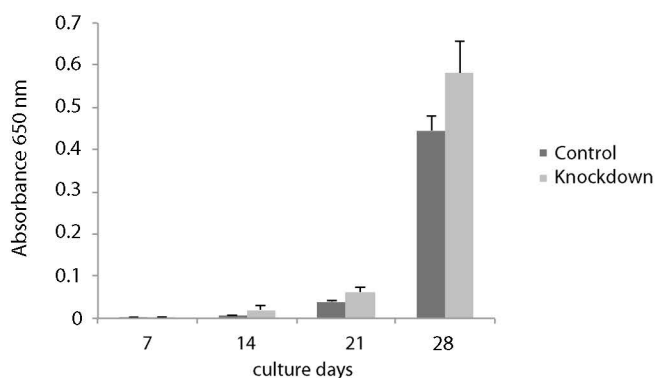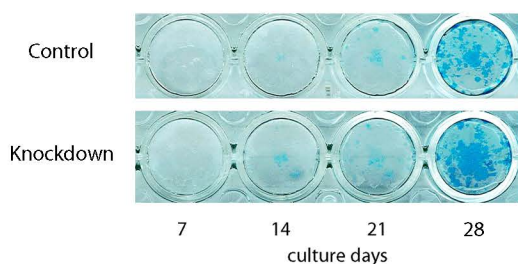
D

Gene		deltaCt	SD	% change
Acan	Ctrl	3.0	0.4	
	KD	2.5	0.2	+41%
Aspn	Ctrl	5.6	0.2	
	KD	5.4	0.1	+10%
Bgn	Ctrl	0.9	0.04	
	KD	0.6	0.06	+17%
Col1a1	Ctrl	-1.3	0.2	
	KD	-1.3	0.08	+3%
Col1a2	Ctrl	-0.5	0.2	
	KD	-0.5	0.05	+4%
Col2a1	Ctrl	3.0	0.08	
	KD	2.1	0.1	+87% *
Col3a1	Ctrl	3.2	0.2	
	KD	2.8	0.04	+31%
Col10a1	Ctrl	8.5	0.3	
	KD	7.6	0.09	+88% *
Dcn	Ctrl	7.0	0.03	
	KD	6.0	0.3	+102% *
Fmod	Ctrl	8.0	0.3	
	KD	7.0	0.1	+106% *
Ihh	Ctrl	10.0	0.2	
	KD	8.5	0.5	+186% *
Lum	Ctrl	8.2	0.1	
	KD	7.8	0.2	+30%
Sox9	Ctrl	5.0	0.3	
	KD	4.2	0.07	+76% *
Vcan	Ctrl	8.1	0.2	
	KD	8.0	0.03	+6%

E

Cell layer   Medium
Ctrl   KD   Ctrl   KD

Collagen I   — 100

Collagen II   — 100

Collagen III   — 130

Aggrecan G3   — 70

Actin   — 40
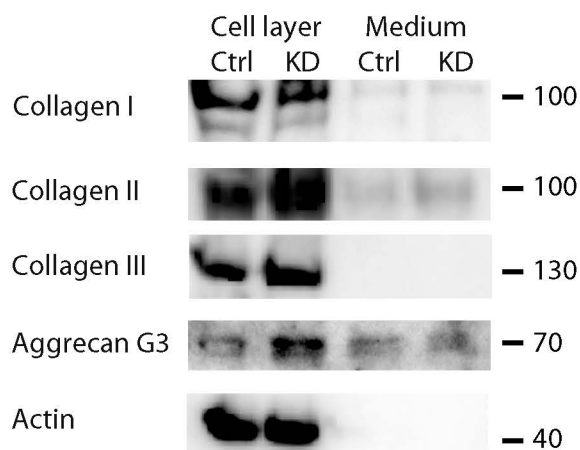
Figure 5

Diseases and Functions

A

Categories	p-Value	Prediction	z-score
angiogenesis	1.31E-07	↑	2.083
accumulation of myeloid cells	5.39E-06	↑	2.176
activation of leukocytes	7.66E-05	↑	2.197
hypertrophy of heart	1.83E-04	↑	2.395
growth of neurites	1.30E-03	↑	2.101
Hypertrophy	1.42E-03	↑	2.309
cell viability of neurons	2.44E-03	↑	2.000
proliferation of connective tissue cells	3.18E-03	↑	2.024
infiltration by lymphocytes	4.51E-03	↑	2.000
organization of cytoplasm	8.30E-03	↑	2.020
formation of cellular protrusions	9.29E-03	↑	2.215
injury of liver	9.50E-06	↓	-2.102
damage of liver	1.20E-05	↓	-2.135

B
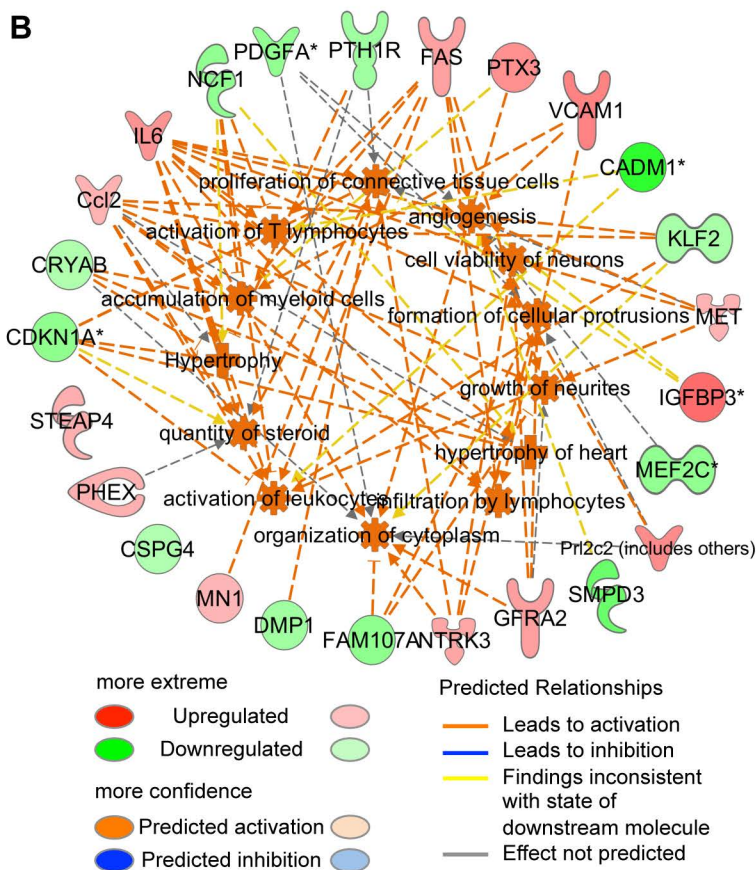
C

Upstream regulator analysis

Genes	z-score	p-value	Prediction
STAT1	4.040	1.20E-14	↑
STAT3	4.272	8.77E-11	↑
NFkB	3.652	9.26E-09	↑
PARP1	2.494	1.54E-07	↑
RELA	2.125	5.20E-06	↑
MET	2.121	1.43E-05	↑
P38 MAPK	2.404	1.78E-04	↑
MAPK14	2.592	7.03E-04	↑
MAPK9	2.770	8.07E-04	↑
MAPK8	2.415	1.73E-03	↑
TGFBR1	2.219	2.48E-03	↑
LEP	2.415	3.46E-02	↑
TGFB1	-2.546	5.20E-06	↓
TNFRSF4	-2.000	9.77E-04	↓