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The Follistatin-like Protein 1 Pathway Is Important for Maintaining Healthy Articular Cartilage

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Objective. We sought to determine whether follistatin-like protein 1 (FSTL1), a protein produced by articular chondrocytes, promotes healthy articular cartilage and prevents chondrocytes from undergoing terminal differentiation to hypertrophic cells.

Methods. In vitro experiments were performed with immortalized human articular chondrocytes. The cells were transduced with a lentivirus encoding human FSTL1 small hairpin RNA or with an adenovirus encoding FSTL1. A quantitative polymerase chain reaction was used for gene expression analysis. Protein expression was assessed by Western blotting. Co-immunoprecipitation was used to identify interacting partners of FSTL1. FSTL1 expression in human articular cartilage was analyzed using confocal microscopy.

Results. Downregulation of FSTL1 expression in transforming growth factor β (TGFβ)-stimulated chondrocyte pellet cultures led to chondrocyte terminal differentiation characterized by poor production of cartilage extracellular matrix and altered expression of genes and proteins involved in cartilage homeostasis, including MMP13, COL10A1, RUNX2, COL2A1, ACAN, Sox9, and phospho-Smad3. We also showed that FSTL1 interacts with TGFβ receptor proteins, Alk1 and endoglin, suggesting a potential mechanism for its effects on chondrocytes. Transduction of chondrocytes with an FSTL1 transgene increased COL2A1 expression, whereas it did not affect MMP13 expression. FSTL1 protein expression was decreased in human osteoarthritic cartilage in situ.

Conclusion. Our data suggest that FSTL1 plays an important role in maintaining healthy articular cartilage and the FSTL1 pathway may represent a therapeutic target for degenerative diseases of cartilage.

INTRODUCTION

Chondrocytes are the only cell type in articular cartilage and play a pivotal role in maintenance of the extracellular matrix (ECM), the primary target of osteoarthritic cartilage degradation. The chondrocytes in osteoarthritis (OA) display dysregulation of anabolic and catabolic processes and are reduced in number (1–3). Numerous in vivo and in vitro studies have shown that the default route of chondrocyte differentiation is terminal differentiation, hypertrophy, and apoptosis (2,4). In healthy articular cartilage, this default route is blocked to obtain permanent cartilage, and chondrocytes remain resting and maintain homeostasis of mature cartilage by a low turnover of ECM molecules. In OA, this block is lifted, resulting in chondrocyte terminal differentiation characterized by altered expression of genes and proteins involved in cartilage homeostasis, including decreased production of ECM proteins (type 2 collagen [COL2] and aggrecan [ACAN]) and increased

production of type 10 collagen (COL10) and matrix metalloproteinase 13 (MMP13), the major type 2 collagen-degrading enzyme (2,5). Understanding how articular chondrocytes undergo phenotypic changes in OA may lead to identification of important players and potential therapeutic targets to block OA cartilage damage and promote its repair.

Follistatin-like protein 1 (FSTL1) is a glycoprotein belonging to a family of secreted proteins acidic and rich in cysteine (SPARC/BM-40/osteonectin) containing both EF-hand calcium-binding and follistatin-like domains (6). In contrast to other family members, the EF-hand calcium-binding domains of FSTL1 are nonfunctional (6). Despite the sequence similarity to follistatin, FSTL1 does not bind activin (7), which suggests that FSTL1 has evolved clearly distinct properties. The biological function of FSTL1 is not fully understood. Studies suggest that FSTL1 plays roles in the regulation of organ development, cell proliferation, differentiation, survival, migration, wound healing, immune cell function,

carcinogenesis, and metastasis (8–13). FSTL1 is produced by mesenchyme-derived cells, including chondrocytes (12,14). Serum FSTL1 levels decrease with age in both humans and mice (15–17), which suggests that FSTL1 plays a role in age-related changes in mesenchyme-derived tissues. As recently published by our group, deletion of FSTL1 in mice results in cartilage hypocellularity (12). Mesenchymal stem cells (MSC) isolated from FSTL1 knockout (KO) embryos exhibit impaired chondrogenic differentiation and displayed alterations in signaling induced by transforming growth factor β (TGFβ), a key chondrogenic factor. Multiple genes known to be involved in chondrogenesis, including those regulated by TGFβ, bone morphogenetic proteins (BMPs), insulin-like growth factors, and Wnt, were found to be dysregulated in FSTL1 KO MSC. Among these genes, reduced expression of SOX9 and COL2A1 is of particular interest because their expression is critical for the early stage of chondrocyte differentiation. We also observed markedly decreased TGFβ-induced phosphorylation of Smad3, a key component of the chondrocyte differentiation pathway. These findings led us to undertake the current study to determine whether FSTL1 promotes healthy articular cartilage and prevents chondrocytes from undergoing terminal differentiation to hypertrophic cells.

MATERIALS AND METHODS

Lentiviral transduction of human articular chondrocytes

Human articular chondrocytes immortalized with the human telomerase gene and HPV oncogenes E6 and E7 (CH-hTERT/E6/E7 cells) were kindly provided by Dr. Aloysius Klingelhutz at the University of Iowa (18). The cells were transduced with a lentivirus encoding human FSTL1 small hairpin RNA (shRNA) (Santa Cruz Biotechnology) or a control lentivirus carrying scrambled shRNA as described (14).

Adenoviral transduction of human articular chondrocytes

A recombinant, E1a-E3–deleted replication defective adenovirus type 5 vector encoding the human FSTL1 gene and the control vector lacking an insert were generated, grown in 293 cells, and purified as described (19). CH-hTERT/E6/E7 cells were infected with adenovirus (1000 viral particles/cell) for 5 days.

Cell culture

For chondrogenic differentiation, 150 000 cells were pelleted in triplicate wells of a V-bottom 96-well polypropylene plate at 300×g for 7 minutes and then cultured in chondrogenic medium (DMEM high glucose [Gibco], 10 ng/ml human TGFβ3 [R&D Systems], 0.1 µM dexamethasone [Tocris], 25 µg/ml L-ascorbate

[Sigma-Aldrich], 1 mM sodium pyruvate [Gibco], 1 × ITS+ Premix [Corning], 100 IU penicillin, and 100 µg/ml streptomycin) at 5% CO_2/37°C. The medium was replaced twice a week. Pellets were harvested for analysis after 1 to 3 weeks. To calculate pellet volumes, the plate was scanned and pellet diameters were measured with ImageJ software. For histochemical analysis, the pellets were cryosectioned and stained with Safranin O/fast green (20).

Analysis of FSTL1 expression in human articular cartilage

In accordance with Institutional Review Board regulations, knee joint tissues from OA patients undergoing total knee replacement (University of Iowa Department of Orthopaedics and Rehabilitation) were deidentified and collected as surgical discards. High Mankin (HM) score (mean = 11.7) vs. low Mankin (LM) score (mean = 2.0) tissue was defined as previously described (21). Frozen cartilage tissue was sectioned at 6 µm in a cryostat with the CryoJane Tape Transfer system (Instrumedics) as previously described (22). FSTL1 was labeled with rabbit anti-human FSTL1 antibody (Pierce) and visualized with Alexa Fluor 647-anti-rabbit IgG (Invitrogen). Nuclei were counterstained with Hoechst 33342 dye (Invitrogen), and the coverslips were mounted onto slides. The slides were imaged using a Zeiss 710 confocal microscope with an oil-immersion objective (63x). Fluorescence intensity was quantified using ImageJ software. The mean fluorescence from at least 30 cells per individual from both LM and HM cartilage was used to calculate the average fluorescence signal.

Quantitative RT-PCR

Chondrocyte pellets or cartilage tissues were frozen on dry ice as soon as they were collected and pulverized using a disposable microhomogenizer. Total RNA extraction was performed using the PureLink RNA Mini Kit (Invitrogen). The SuperScript II Reverse Transcriptase Kit (Invitrogen) was used to make complementary DNA. PCR was performed in a LightCycler480 (Roche) using the Power SYBR Green PCR Master Mix (Applied

Table 1. Primer sequences

| Human Gene	Sequence of Primer Pair
ACAN	forward 5'-TCGAGGACAGCGAGGCC-3' reverse 5'-TCGAGGGTGTAGCGTGTAGAGA-3'
COL2A1	forward 5'-TGGACGCCATGAAGGTTTTCT-3' reverse 5'-TGGGAGCCAGATTGTCATCTC-3'
COL10A1	forward 5'-ATGCTGCCACAAATACCCTTT-3' reverse 5'-GGTAGTGGGCCTTTTATGCCT-3'
FSTL1	forward 5'-CGATGGACACTGCAAAGAGA-3' reverse 5'-CCAGCCATCTGGAATGATCT-3'
GAPDH	forward 5'-TTCACCACCATGGAGAAGGC-3 reverse 5'-GGCATGGACTGTGGTCATGA-3'
MMP13	forward 5'-CCAGACTTCACGATGGCATTG-3' reverse 5'-GGCATCTCCTCCATAATTTGGC-3'
RUNX2	forward 5'-CCGCCTCAGTGATTTAGGGC-3' reverse 5'-GGGTCTGTAATCTGACTCTGTCC-3'
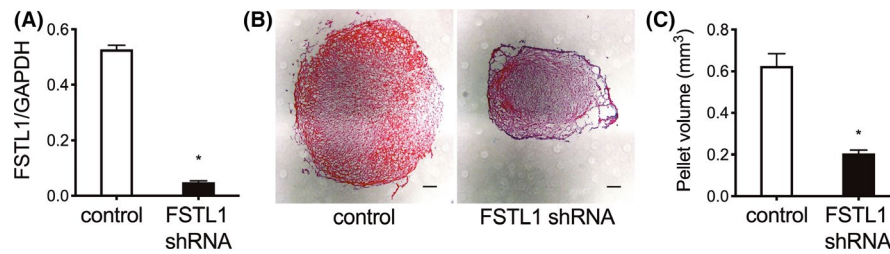
Figure 1. Suppression of follistatin-like protein 1 (FSTL1) in human articular chondrocytes reduces cartilage proteoglycan content and volume. CH-hTERT/E6/E7 chondrocytes were transduced with lentivirus encoding FSTL1 short hairpin RNA (shRNA) or scrambled shRNA (control). Chondrocyte differentiation was performed in the presence of transforming growth factor β. **A**, FSTL1 expression was assayed by real-time PCR at day 0 of differentiation. The mean + SEM is shown (n = 3, *P = 0.004, two-tailed *t* test). **B**, After 21 days, pellets were sectioned and stained with Safranin O/Fast green to demonstrate proteoglycan content (scale bars, 100 μm). The representative data shown are from one of three experiments. **C**, The plate was scanned and pellet diameters were measured with ImageJ software and used to calculate pellet volumes. The mean + SEM is shown (n = 3, *P = 0.0055, two-tailed *t* test).

Biosystems) as described (23). The copy number (number of transcripts) of amplified product was calculated from a standard curve obtained by plotting the known input concentrations of plasmid DNA. Expression levels of each gene were normalized to GAPDH (see Table 1 for primer sequences).

Western blotting

Chondrocyte pellets were lysed in 2× SDS-PAGE loading buffer and sonicated. SDS-PAGE and immunoblotting were performed as described (23). Goat polyclonal antibodies were used to detect human FSTL1 and MMP13 (R&D Systems). Phospho-Smad3, Sox9, and actin were detected with rabbit polyclonal antibodies (Cell Signaling). Blots were developed using horseradish peroxidase–conjugated secondary antibodies (Thermo) with a chemiluminescent substrate (Thermo). Membranes were scanned

by LAS4000 imaging system (Fuji), and images were analyzed with Multi Gauge software.

Co-immunoprecipitation

HEK 293T cells were transfected with empty expression vector, or plasmids encoding mouse FLAG-FSTL1, Birch profilin (BP)-epitope–tagged mouse activin receptor-like kinase 1 (Alk1), and endoglin. Also, co-transfections of FLAG-FSTL1 with TGFβ receptor proteins were performed. Forty-eight hours after transfection, cells were lysed in buffer containing 1% Triton X-100. FLAG-FSTL1 was immunoprecipitated from clarified lysates using anti-FLAG antibody (Sigma). Immunoprecipitates were resolved by SDS-PAGE. FLAG-FSTL1 was visualized by Western blotting with anti-FSTL1 antibody. Alk1 and endoglin were visualized with anti-BP antibody.
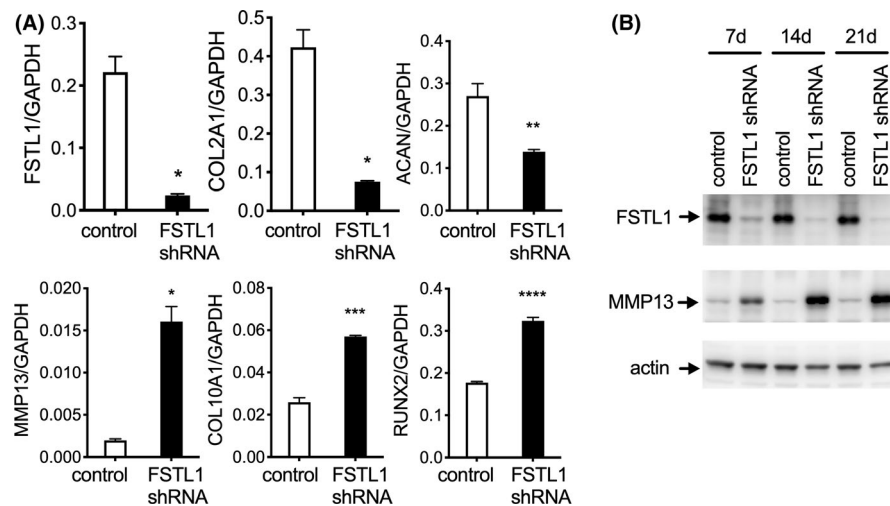

Figure 2. Follistatin-like protein 1 (FSTL1) expression regulates chondrocyte phenotype. CH-hTERT/E6/E7 chondrocytes were transduced with lentivirus encoding FSTL1 short hairpin RNA shRNA) or scrambled shRNA (control). Chondrocyte differentiation was performed in the presence of transforming growth factor β. **A**, After 21 days of culture, FSTL1, COL2A1, ACAN, MMP13, COL10A1, and RUNX2 were assayed by real-time PCR. The mean + SEM is shown (n = 3; FSTL1 *P = 0.01, COL2A1 *P = 0.01, ACAN **P = 0.028, MMP13 *P = 0.01, COL10A1 ***P = 0.0015, RUNX2 ****P = 0.0039; two-tailed *t* test). **B**, After 7, 14, or 21 days of culture, cell lysates were assayed by Western blotting for FSTL1, MMP13, or actin.
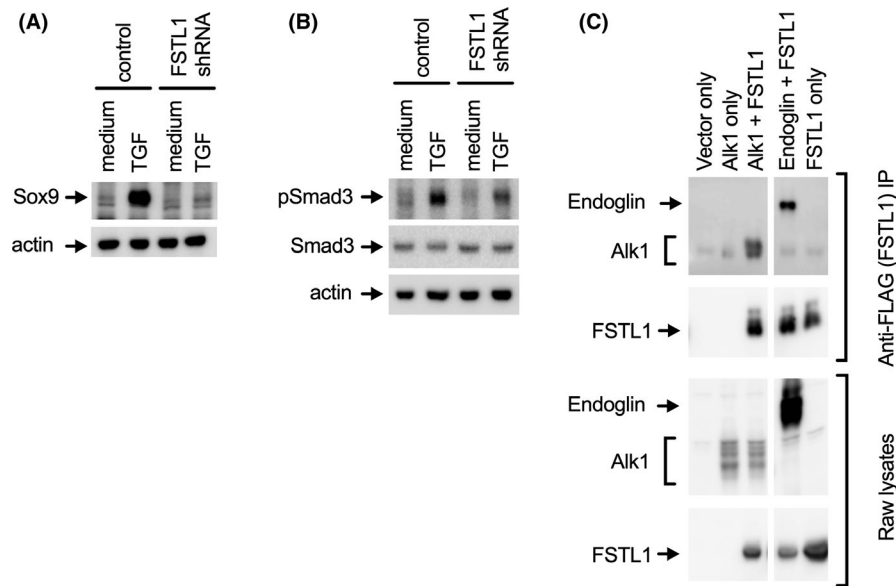
Figure 3. Follistatin-like protein 1 (FSTL1) regulates transforming growth factor β (TGFβ) signaling. CH-hTERT/E6/E7 chondrocytes were transduced with lentivirus encoding FSTL1 short hairpin RNA (shRNA) or scrambled shRNA (control). Chondrocyte differentiation was performed in the presence of TGFβ. After 3 days of culture, cell lysates were assayed by Western blotting for Sox9 (**A**) or phosphor-(p) and total Smad3 (**B**). Duplicate blots were probed for actin to confirm equivalent lane loading. The results shown are from one representative experiment of three performed. C, HEK 293T cells were transfected with empty expression vector or plasmids encoding FLAG-FSTL1 and BP-epitope–tagged Alk1 or endoglin. FLAG-FSTL1 was immunoprecipitated from lysates using anti-FLAG antibody. FSTL1 was visualized with anti-FSTL1 antibody. Alk1 and endoglin were visualized with anti-BP antibody. The results shown are from one representative experiment of three performed.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 8 software (GraphPad). The parametric Student's *t* test (two-tailed) was used to assess the significance of differences between two groups. Levels of FSTL1 expression in human cartilage were compared using the Mann-Whitney *U* test. Correlation analysis was performed by the Pearson correlation test. The mean + SEM is shown in the figures. *P* values < 0.05 were considered statistically significant.

RESULTS

FSTL1 prevents chondrocyte terminal differentiation

To explore the potential role of FSTL1 in articular cartilage, we used a line of immortalized human articular chondrocytes (18). In the presence of TGFβ, these cells can differentiate into chondrocytes and form cartilage pellets in culture (24). As shown in Supplementary Figure 1, stimulation of cells with TGFβ led to a marked increase in expression of COL2A1 and ACAN, two genes associated with healthy articular cartilage. To reduce FSTL1 expression, the cells were transduced with a lentivirus encoding human FSTL1 shRNA. Cells transduced with a virus carrying scrambled shRNA were used as a control. FSTL1 shRNA reduced FSTL1 expression to less than 10% of that in control cells (Figure 1A). FSTL1

knockdown had no effect on expression of ACAN, COL2A1, COL10A1, MMP13, and RUNX2 in unstimulated pellet cultures (Supplementary Figure 2). Upon stimulation with TGFβ, FSTL1-deficient chondrocytes produced markedly less ECM proteoglycan, as demonstrated by poor Safranin O staining (Figure 1B) and smaller pellet volumes (Figure 1C). FSTL1-deficient chondrocytes had reduced expression of COL2A1 and ACAN (Figure 2A). At the same time, we observed increased expression of genes associated with the development of terminally differentiated hypertrophic/OA-like chondrocytes, including MMP13, COL10A, and RUNX2 (Figure 2A). Because MMP13 is a pivotal marker of chondrocyte hypertrophy and OA progression, we next performed Western blotting to examine the expression of MMP13 at the protein level. MMP13 protein expression was dramatically upregulated by FSTL1 shRNA treatment (Figure 2B).

Critical role of FSTL1 in the regulation of TGFβ signaling

To gain deeper insight into the regulatory mechanisms underlying the effects of FSTL1 on chondrocytes, protein extracts from control and FSTL1 shRNA cells were analyzed for phospho-Smad3 and Sox9 by Western blotting. TGFβ-induced Sox9, a crucial transcription factor for healthy articular chondrocytes (25–27), was decreased in FSTL1-deficient cells (Figure 3A). Also, a marked reduction in phosphorylation of Smad3, which blocks
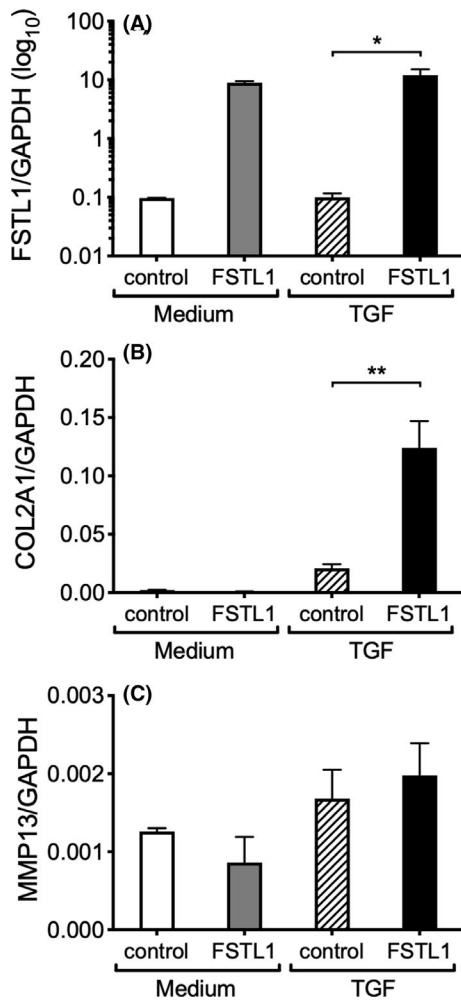
Figure 4. Overexpression of Follistatin-like protein 1 (FSTL1) enhances COL2A1 gene expression. CH-hTERT/E6/E7 chondrocytes were infected with adenovirus encoding FSTL1 (FSTL1) or a control virus lacking a transgene (control). Chondrogenic differentiation was performed in a 21-day pellet culture with transforming growth factor β (TGFβ). FSTL1 (**A**), COL2A1 (**B**), and MMP13 (**C**) expression was assessed by real-time PCR. The mean + SEM is shown (n = 3; FSTL1 *P = 0.014, COL2A1 **P = 0.0098, MMP13 P = 0.55; two-tailed t test).

chondrocyte terminal differentiation (28), was observed at 30 minutes after TGFβ treatment (Supplementary Figure 3) and in long-term (3 days) chondrocyte pellet cultures (Figure 3B). The results of these experiments demonstrate that altering FSTL1 mRNA levels leads to a change in TGFβ signaling. We hypothesized that FSTL1 binds to proteins important in the regulation of TGFβ signaling and cartilage homeostasis. Putative binding partners that may interact with FSTL1 include a number of members of the TGFβ superfamily or their receptors (29–31). Next, we co-transfected 293T cells with FLAG-FSTL1 and TGFβ receptor proteins, Alk1 and endoglin, and then immunoprecipitated FLAG-FSTL1. The immunoprecipitates were evaluated by Western blotting. As shown in Figure 3C, Alk1 and endoglin co-immunoprecipitated with FLAG-FSTL1.
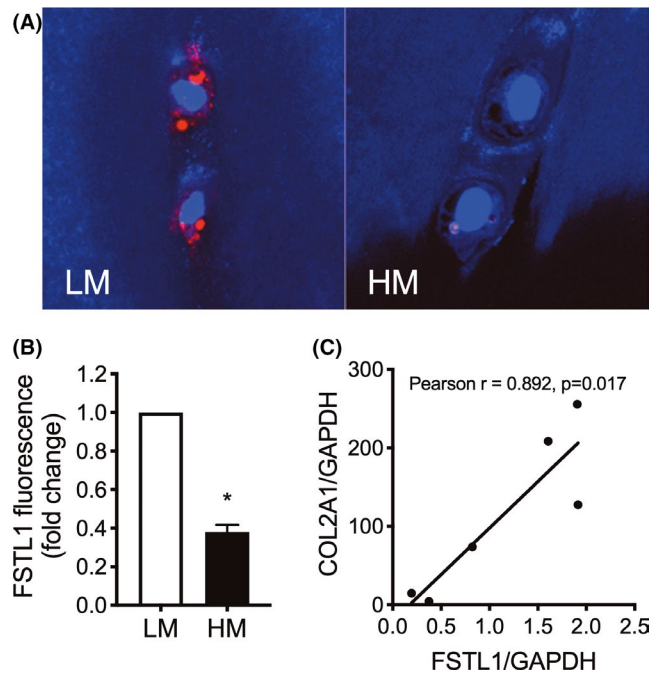

Figure 5. Follistatin-like protein 1 (FSTL1) expression is reduced in OA articular cartilage. Cryosections from healthy-appearing, low Mankin (LM) score and diseased-appearing, high Mankin (HM) score tissues were stained with rabbit anti-human FSTL1 followed by Alexa Fluor 647-anti-rabbit IgG (red). Hoechst 33342 was used to counterstain nuclei (blue). Slides were imaged by confocal microscopy using a ×63 oil-immersion objective. **A**, Images are representative of at least 15 microscope fields from one of four donors. **B**, The mean + SEM of the fold change in the average fluorescence signal (LM vs HM) is shown (*P < 0.01, Mann-Whitney U test). **C**, COL2A1 and FSTL1 expression in tissues with LM and HM scores (LM, n = 3; HM, n = 3) were assayed by real-time PCR and normalized to GAPDH. Each circle represents an individual data point. The Pearson's correlation coefficient (r) and P value are shown.

Overexpressing FSTL1 promotes COL2A1 expression

We next evaluated whether overexpression of FSTL1 impacts anabolic and catabolic activities in chondrocytes. Overexpressing FSTL1 using an adenovirus encoding FSTL1 vector enhanced TGFβ-induced COL2A1 gene expression by over 5-fold (Figure 4A,B). In contrast, the expression of MMP13 was essentially unaffected (Figure 4C).

FSTL1 expression is reduced in human OA cartilage

The effects of FSTL1 on chondrocytes demonstrated in vitro suggested changes in expression of FSTL1 in human OA cartilage. To examine this hypothesis, we obtained cartilage tissue from OA patients undergoing total knee replacement (University of Iowa Department of Orthopaedics and Rehabilitation) and compared FSTL1 expression in healthy-appearing versus diseased-appearing

tissue, using the Mankin scoring system to define diseased versus healthy cartilage (21).

FSTL1 expression in chondrocytes of high Mankin score cartilage was significantly lower than that in the chondrocytes of low Mankin score cartilage (Figure 5). Also, we assayed tissues with low and high Mankin scores for COL2A1 and FSTL1 expression by real-time PCR. A significant positive correlation was observed between decreasing FSTL1 expression levels and a decrease in levels of COL2A1 expression ($r = 0.892$, $P = 0.017$).

DISCUSSION

The current study provides compelling evidence that FSTL1 promotes healthy articular cartilage. We found that FSTL1 inhibits TGFβ-mediated terminal differentiation of chondrocytes, promotes production of ECM proteins, and inhibits production of tissue-destructive matrix metalloproteinases. In situ, FSTL1 protein expression was decreased in human osteoarthritic cartilage.

Microarray analysis of MSC gene expression in FSTL1-deficient mice, recently reported by our group (12), revealed dysregulation of multiple genes known to be involved in the maintenance of normal articular chondrocyte phenotype. In the current study, we evaluated the effect of FSTL1 on expression of several other phenotypic markers that have been shown to differentiate healthy versus hypertrophic chondrocytes (32–34). These include: ACAN, an essential cartilage proteoglycan and a marker of healthy articular chondrocytes (35); COL10A1, not normally expressed in healthy human articular cartilage but whose expression is detected at protein and mRNA level in human OA cartilage (25,36–38); MMP13, which is highly elevated in OA cartilage (39–42); Sox9, a crucial transcription factor for chondrocytes that is down regulated in OA cartilage (25–27,43); Smad3, which blocks chondrocyte terminal differentiation (44); and Runx2, a master transcription factor that controls chondrocyte hypertrophic differentiation (45–47).

In TGFβ-stimulated chondrocytes, suppression of FSTL1 led to reduced ACAN, COL2A1, increased COL10A1 and MMP13, reduced Sox9 and phospho-Smad3, and increased RUNX2. Taken together, these results demonstrate that FSTL1 blocks chondrocyte terminal differentiation in the presence of TGFβ. TGFβ stimulates the initial stages of chondrogenesis but represses chondrocyte terminal differentiation (2). Our data suggest that FSTL1 is involved in phenotypic stabilization of the prehypertrophic chondrocytes mediated by TGFβ. Because chondrogenic differentiation is dependent on the expression of Sox9 and FSTL1 regulates Sox9 status, it is also possible that FSTL1-deficient cells fail to differentiate into chondrocytes.

It has been reported that FSTL1 may interact with key chondrogenic mediators, proteins of the TGFβ superfamily, or their receptors (29–31). It has also been suggested that FSTL1 inhibits the formation of the BMP ligand-receptor complex (29). However, it is unlikely that FSTL1 simply functions as an inhibitor of BMP because overexpression of FSTL1 in mice (9), zebrafish

(48), or frogs (49) did not result in developmental abnormalities. Also, despite the fact that FSTL1 has the capacity to interact with TGFβ (29,31), its impact on downstream signaling has not yet been demonstrated. Our results show that chondrocytes underexpressing FSTL1 display alterations in TGFβ-induced signaling. We found that Alk1 and endoglin coprecipitate with FSTL1. These results imply that FSTL1 acts by binding Alk1 and endoglin, potentially altering assembly of the BMPRII complex and cell surface expression of BMPRII. This, in turn, would block subsequent signaling cascade, leading to activation of RUNX2 in response to TGFβ. Studies to show possible effects of FSTL1 on assembly of the BMP receptor complex are currently underway.

In the current study, we showed that overexpression of FSTL1 can increase COL2A1 expression in human chondrocytes. We hypothesize that even if an OA chondrocyte has a reduced expression of ECM proteins, it might still be possible for that chondrocyte to increase its expression of ECM proteins under the right stimulation conditions. FSTL1 overexpression may also lead to a stronger commitment to chondrogenesis among the cells in the population. Interestingly, results from our experiments showed that the increased expression of FSTL1 did not induce expression of MMP13. This suggests that overexpression of FSTL1 in normal articular chondrocytes does not initiate differentiation toward an OA/hypertrophic phenotype.

In a recently published study (50), FSTL1 increased the expression of inflammatory cytokines (IL-1β, TNFα, IL-6) and metalloproteinases, including MMP13, in rat chondrocytes. The results of this work are in contradiction with our data. A likely explanation for this might be explained by the difference of their experimental setup. In the study by Hu et al (50), the authors used dedifferentiated chondrocytes in monolayer culture and incubated cells with FSTL1 without TGFβ for 24 hours. In the current study, we utilized a markedly different experimental model. We examined effects of FSTL1 underexpression on cells undergoing chondrocyte differentiation in the presence of TGFβ in long-term (up to 3 weeks) pellet culture, which is a more relevant approach for exploring the molecular mechanisms underlying chondrocyte differentiation and activation.

Our observations may have clinical relevance. FSTL1 protein expression is reduced in human OA cartilage, a finding supporting the observation of others that chondrocytes express significantly higher amounts of FSTL1 in macroscopically healthy cartilage when compared with diseased tissue (51). There are currently no disease-modifying OA drugs, and identifying potential disease-modifying targets would represent an exciting advance in the field. The results of our work suggest that increasing FSTL1 expression in articular chondrocytes would slow or reverse OA progression.

In the past decade, a number of studies have been published suggesting a role for FSTL1 in inflammatory response (13). However, it is not completely understood whether FSTL1 induces or inhibits inflammation; both pro- and anti-inflammatory functions for

FSTL1 have been reported (13). Murakami et al (52) proposed that the activation of multiple receptors might explain pro- and anti-in-flammatory activities of FSTL1. The dual action of FSTL1 may also depend on its concentration, tissue microenvironment, and presence of various regulatory molecules during different stages of inflammatory response.

Wang et al (53) showed that FSTL1 is weakly expressed in the chondrocytes of articular cartilage obtained from patients with OA, whereas it was overexpressed in synovial and capillary endothelial cells of the synovial tissue. The concentrations of FSTL1 in serum and synovial fluid were significantly higher in OA patients than in respective control subjects (53). OA is a complex disease characterized by chronic inflammation involving the whole synovial joint; IL-1β and TNFα are key proinflammatory cytokines driving OA progression via induction of protease synthesis (54). Both of these cytokines can also induce FSTL1 expression in various cells comprising synovium, joint capsule, and subchondral bones (13,22). Thus, FSTL1 might serve as a biomarker reflecting the severity of joint damage. The results of this study do not rule out our hypothesis that FSTL1 may have potential beneficial effects on articular cartilage in the early stage of OA.

In summary, our data suggest that FSTL1 plays an important role in maintaining healthy articular cartilage and the FSTL1 pathway may represent a therapeutic target for degenerative diseases of cartilage.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

All authors drafted the article, revised it critically for important intellectual content, approved the final version to be published, and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Chaly, Hostager, Hirsch.
Acquisition of data. Chaly, Hostager, Smith.
Analysis and interpretation of data. Chaly, Hostager, Smith, Hirsch.

REFERENCES

1. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 1998;41:1632–8.

2. Van der Kraan PM, van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? [research support]. Osteoarthritis Cartilage 2012;20:223–32.

3. Grogan SP, D'Lima DD. Joint aging and chondrocyte cell death. Int J Clin Rheumtol 2010;5:199–214.

4. Zhong L, Huang X, Karperien M, Post JN. The regulatory role of signaling crosstalk in hypertrophy of MSCs and human articular chondrocytes. Int J Mol Sci 2015;16:19225–47.

5. Akkiraju H, Nohe A. Role of chondrocytes in cartilage formation, progression of osteoarthritis and cartilage regeneration. J Dev Biol 2015;3:177–92.

6. Hambrock HO, Kaufmann B, Müller S, Hanisch FG, Nose K, Paulsson M, et al. Structural characterization of TSC-36/Flik: analysis of two charge isoforms. J Biol Chem 2004;279:11727–35.

7. Kawabata D, Tanaka M, Fujii T, Umehara H, Fujita Y, Yoshifuji H, et al. Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis. Arthritis Rheum 2004;50:660–8.

8. Oshima Y, Ouchi N, Sato K, Izumiya Y, Pimentel DR, Walsh K. Follistatin-like 1 is an Akt-regulated cardioprotective factor that is secreted by the heart. Circulation 2008;117:3099–108.

9. Liu S, Shen H, Xu M, Liu O, Zhao L, Liu S, et al. FRP inhibits ox-LDL-induced endothelial cell apoptosis through an Akt-NF-κB-Bcl-2 pathway and inhibits endothelial cell apoptosis in an apoE-knockout mouse model. Am J Physiol Endocrinol Metab 2010;299:E351–63.

10. Trojan L, Schaaf A, Steidler A, Haak M, Thalmann G, Knoll T, et al. Identification of metastasis-associated genes in prostate cancer by genetic profiling of human prostate cancer cell lines. Anticancer Res 2005;25:183–91.

11. Sundaram GM, Common JE, Gopal FE, Srikanta S, Lakshman K, Lunny DP, et al. 'See-saw' expression of microRNA-198 and FSTL1 from a single transcript in wound healing. Nature 2013;495:103–6.

12. Chaly Y, Blair HC, Smith SM, Bushnell DS, Marinov AD, Campfield BT, et al. Follistatin-like protein 1 regulates chondrocyte proliferation and chondrogenic differentiation of mesenchymal stem cells. Ann Rheum Dis 2015;74:1467–73.

13. Chaly Y, Hostager B, Smith S, Hirsch R. Follistatin-like protein 1 and its role in inflammation and inflammatory diseases. Immunol Res 2014;59:266–72.

14. Chaly Y, Marinov AD, Oxburgh L, Bushnell DS, Hirsch R. FSTL1 promotes arthritis in mice by enhancing inflammatory cytokine/chemokine expression. Arthritis Rheum 2012;64:1082–8.

15. Gorelik M, Fall N, Altaye M, Barnes MG, Thompson SD, Grom AA, et al. Follistatin-like protein 1 and the ferritin/erythrocyte sedimentation rate ratio are potential biomarkers for dysregulated gene expression and macrophage activation syndrome in systemic juvenile idiopathic arthritis. J Rheumatol 2013;40:1191–9.

16. Gorelik M, Wilson DC, Cloonan YK, Shulman ST, Hirsch R. Plasma follistatin-like protein 1 is elevated in Kawasaki disease and may predict coronary artery aneurysm formation. J Pediatr 2012;161:116–9.

17. Chaly Y, Fu Y, Marinov A, Hostager B, Yan W, Campfield B, et al. Follistatin-like protein 1 enhances NLRP3 inflammasome-mediated IL-1β secretion from monocytes and macrophages. Eur J Immunol 2014;44:1467–79.

18. Martin JA, Mitchell CJ, Klingelhutz AJ, Buckwalter JA. Effects of telomerase and viral oncogene expression on the in vitro growth of human chondrocytes. J Gerontol A Biol Sci Med Sci 2002;57:B48–53.

19. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. J Virol 1997;71:1842–9.

20. Schmitz N, Laverty S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. Osteoarthritis Cartilage 2010;18:S113–6.

21. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 1971;53:523–37.

22. Wilson DC, Marinov AD, Blair HC, Bushnell DS, Thompson SD, Chaly Y, et al. Follistatin-like protein 1 is a mesenchyme-derived

inflammatory protein and may represent a biomarker for systemic-onset juvenile rheumatoid arthritis. Arthritis Rheum 2010;62:2510–6.

23. Clutter SD, Wilson DC, Marinov AD, Hirsch R. Follistatin-like protein 1 promotes arthritis by up-regulating IFN-γ. J Immunol 2009;182: 234–9.

24. Goldring MB. Human chondrocyte cultures as models of cartilage-specific gene regulation. Methods Mol Med 2005;107:69–95.

25. Brew CJ, Clegg PD, Boot-Handford RP, Andrew JG, Hardingham T. Gene expression in human chondrocytes in late osteoarthritis is changed in both fibrillated and intact cartilage without evidence of generalised chondrocyte hypertrophy. Ann Rheum Dis 2010;69: 234–40.

26. Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 2001;9 Suppl A:S69–75.

27. Orfanidou T, Iliopoulos D, Malizos KN, Tsezou A. Involvement of SOX-9 and FGF-23 in RUNX-2 regulation in osteoarthritic chondrocytes. J Cell Mol Med 2009;13:3186–94.

28. Yang X, Chen L, Xu X, Li C, Huang C, Deng CX. TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 2001;153:35–46.

29. Geng Y, Dong Y, Yu M, Zhang L, Yan X, Sun J, et al. Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development. Proc Natl Acad Sci U S A 2011;108:7058–63.

30. Sylva M, Li VS, Buffing AA, van Es JH, van den Born M, van der Velden S, et al. The BMP antagonist follistatin-like 1 is required for skeletal and lung organogenesis. PLoS One 2011;6:e22616.

31. Tanaka M, Murakami K, Ozaki S, Imura Y, Tong XP, Watanabe T, et al. DIP2 disco-interacting protein 2 homolog A (Drosophila) is a candidate receptor for follistatin-related protein/follistatin-like 1– analysis of their binding with TGF-β superfamily proteins. FEBS J 2010;277:4278–89.

32. Alvarez J, Balbín M, Santos F, Fernández M, Ferrando S, López JM. Different bone growth rates are associated with changes in the expression pattern of types II and X collagens and collagenase 3 in proximal growth plates of the rat tibia. J Bone Miner Res 2000;15:82–94.

33. Kirsch T, von der Mark K. Remodelling of collagen types I, II and X and calcification of human fetal cartilage. Bone Miner 1992;18:107–17.

34. Nurminskaya M, Linsenmayer TF. Identification and characterization of up-regulated genes during chondrocyte hypertrophy. Dev Dyn 1996;206:260–71.

35. Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Mol Cell Biol 2008;28:4999–5013.

36. Aigner T, Fundel K, Saas J, Gebhard PM, Haag J, Weiss T, et al. Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. Arthritis Rheum 2006;54:3533–44.

37. Von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K, et al. Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806–11.

38. Walker GD, Fischer M, Gannon J, Thompson RC Jr, Oegema TR Jr. Expression of type-X collagen in osteoarthritis. J Orthop Res 1995;13:4–12.

39. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, et al. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534–45.

40. Fukui N, Miyamoto Y, Nakajima M, Ikeda Y, Hikita A, Furukawa H, et al. Zonal gene expression of chondrocytes in osteoarthritic cartilage. Arthritis Rheum 2008;58:3843–53.

41. Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor β. Arthritis Rheum 1997;40:1653–61.

42. Shlopov BV, Gumanovskaya ML, Hasty KA. Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis. Arthritis Rheum 2000;43:195–205.

43. Hattori T, Müller C, Gebhard S, Bauer E, Pausch F, Schlund B, et al. SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. Development 2010;137:901–11.

44. Alvarez J, Serra R. Unique and redundant roles of Smad3 in TGF-β–mediated regulation of long bone development in organ culture. Dev Dyn 2004;230:685–99.

45. Wang X, Manner PA, Horner A, Shum L, Tuan RS, Nuckolls GH. Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. Osteoarthritis Cartilage 2004;12:963–73.

46. Hecht J, Seitz V, Urban M, Wagner F, Robinson PN, Stiege A, et al. Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(-/-) mouse model. Gene Expr Patterns 2007;7:102–12.

47. Higashikawa A, Saito T, Ikeda T, Kamekura S, Kawamura N, Kan A, et al. Identification of the core element responsive to runt-related transcription factor 2 in the promoter of human type X collagen gene. Arthritis Rheum 2009;60:166–78.

48. Dal-Pra S, Fürthauer M, Van-Celst J, Thisse B, Thisse C. Noggin1 and Follistatin-like2 function redundantly to Chordin to antagonize BMP activity. Dev Biol 2006;298:514–26.

49. Okabayashi K, Shoji H, Onuma Y, Nakamura T, Nose K, Sugino H, et al. cDNA cloning and distribution of the Xenopus follistatin-related protein. Biochem Biophys Res Commun 1999;254:42–8.

50. Hu PF, Ma CY, Sun FF, Chen WP, Wu LD. Follistatin-like protein 1 (FSTL1) promotes chondrocyte expression of matrix metalloproteinase and inflammatory factors via the NF-κB pathway. J Cell Mol Med 2019;23:2230–7.

51. Stenberg J, Rüetschi U, Skiöldebrand E, Kärrholm J, Lindahl A. Quantitative proteomics reveals regulatory differences in the chondrocyte secretome from human medial and lateral femoral condyles in osteoarthritic patients. Proteome Sci 2013;11:43.

52. Murakami K, Tanaka M, Usui T, Kawabata D, Shiomi A, Iguchi-Hashimoto M, et al. Follistatin-related protein/follistatin-like 1 evokes an innate immune response via CD14 and toll-like receptor 4. FEBS Lett 2012;586:319–24.

53. Wang Y, Li D, Xu N, Tao W, Zhu R, Sun R, et al. Follistatin-like protein 1: a serum biochemical marker reflecting the severity of joint damage in patients with osteoarthritis. Arthritis Res Ther 2011;13:R193.

54. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage 2013;21:16–21.