Healthy and osteoarthritic synovial fibroblasts produce ADAMTS-4, -5, -7 and -12: Induction by IL-1β and fibronectin and contribution to cartilage damage

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Abstract

Current description of osteoarthritis includes the involvement of synovial inflammation. Studies contributing to understanding the mechanisms of cross-talk and feed-back among the joint tissues could be relevant to the development of therapies that block disease progression. During osteoarthritis synovial fibroblasts exposed to anomalous mechanical forces and to an inflammatory microenvironment, release factors that mediate tissue damage and perpetuate inflammation, such as ADAMTS metalloproteinases. Thus, we studied the production of ADAMTS by synovial fibroblasts and their contribution to cartilage degradation. Moreover, we analyzed the implication of two mediators present in the osteoarthritis joint, IL-1β as pro-inflammatory cytokine, and 45 kDa fibronectin fragments (Fn-fs) as products of matrix degradation. We reported that synovial fibroblasts constitutively express and release ADAMTS-4, -5, -7 and -12. Despite the contribution of both mediators to the stimulation of Runx2 and Wnt/β-catenin signaling pathways, as well as to ADAMTS expression, promoting the degradation of aggrecan and cartilage oligomeric matrix protein (COMP) from cartilage, they were Fn-fs rather than IL-1β, which played the major pathologic role in osteoarthritis, contributing to the maintenance of the disease. Moreover, higher levels of ADAMTS-4 and -7 and a specific regulation of ADAMTS-12 were observed in osteoarthritis, turning them into new potential targets for the design of therapies. Therefore, synovial fibroblasts provide the biochemical tools to the chronicity and destruction of the osteoarthritic joints.

Introduction

Osteoarthritis (OA), one of the leading causes of substantial physical and psychological disability worldwide, is a complex disease with a prevalence of more than 70% in the population over 55 years.[1, 2] Current description of OA includes not only the remodeling of articular cartilage and adjacent bone, but also the involvement of synovial inflammation which is characterized by thickening of synovium or, indirectly, by joint effusion. Synovial membrane inflammation and proliferation in OA joints trigger the production of cytokines and proteinases that damage connective tissues, including the cartilage.[3, 4] In this sense, research on inhibitory mediators of synovial activation could identify ways to avoid the progressive cartilage degradation and functional impairment. The role of synovial fibroblasts (SF) as active drivers of joint destruction in rheumatoid arthritis is well established,[5] but their behavior in healthy subjects and OA patients is poorly understood. It has been described that OA-SF exposed to anomalous mechanical forces and to an inflammatory microenvironment, release factors such as the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), that mediate tissue damage and perpetuate inflammation.[2, 6-9] Therefore, studies contributing to a better understanding of the cross-talk and feed-back mechanisms among the joint tissues could be relevant to the development of new therapies able to block disease progression.

The aim of this study was to elucidate the role of SF in the cartilage joint degradation in OA patients through the production of ADAMTS and to characterize these metalloproteinases in HD-SF. We have mapped the expression and function of aggrecanases ADAMTS-4 and -5, which degrade aggrecan, one of the main components of the cartilage extracellular matrix (ECM) that facilitates cartilage to

resist compression.[10] We have also characterized ADAMTS-7 and -12, involved in destruction of cartilage oligomeric matrix protein (COMP), a non-collagenous component of the cartilage ECM which contributes to its assembly and to the cartilage integrity.[11] Moreover, we studied the physiopathological effect of two mediators present in OA joint microenvironment: the catabolic cytokine interleukin-1β (IL-1β), and the 45 kDa fibronectin fragments (Fn-fs) as products of cartilage ECM degradation.[12-15] This study is the first to report the expression and release of ADAMTS-7 and -12 by SF from HD and OA patients, both constitutively and after IL-1β or Fn-fs stimulation. Besides, the capacity of SF to attach and degrade the cartilage ECM, generating glycosaminoglycans (GAGs) and releasing COMP is also described. Finally, we study the activation of Runx2 and β-catenin, two signaling pathways related to ADAMTS expression.[16-18] Our study reports that SF activated by mediators present in the joint, such as a proinflammatory cytokines and Fn-fs, release ADAMTS which contribute to the maintenance of cartilage destruction in osteoarthritic patients.

Materials and Methods

Patients and synovial fibroblasts cultures

Synovial tissue was obtained from 20 active OA patients (16 women and 4 men) aged between 48 and 87, at the time of knee prosthetic replacement surgery. Patients had advanced disease and were diagnosed of primary OA, excluding trauma, inflammatory disease, and other structural causes of secondary OA. Control samples from HD were obtained from 4 patients (2 women and 2 men) aged between 35 and 72, at the time of knee arthroscopic evaluation. These patients were diagnosed with meniscopathy caused by trauma to the knee or sports injury,

excluding inflammatory and rheumatic diseases. The study was performed according to the recommendations of the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of the Hospital La Princesa (Madrid, Spain). All biopsy samples were obtained after subjects gave informed consent.

SF cultures were established by explant growth of synovial biopsies, cultured in Dulbecco's modified Eagle's medium (DMEM) with 25Mm HEPES and 4.5 g/l glucose, completed with 10% heat-inactivated fetal bovine serum (FBS) (Lonza Ibérica S.A.U., Barcelona, Spain), 1% L-glutamine and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA), at 37°C and 5% CO_2. After 3 passages, residual contamination by macrophages was avoided, previously assessed by flow cytometry analysis of SF with a purity of 95 %.[19] Monocultures of SF were used for experiments until passage 8. Despite the use of cells at varying passage numbers, all comparisons within a same experimentation were made on SF at an identical passage number and at 80-90% confluent.

For treatments, HD- and OA-SF were cultured in serum-free DMEM with 1% L-glutamine and 1% antibiotic-antimycotic, in the absence (untreated) or presence of the following agents: 10ng/ml IL-1β (ImmunoTools) or 10 nM Fn-fs 45 kDa (Sigma-Aldrich, St Louis, MO, USA).

RNA extraction and RT-qPCR for ADAMTS gene expression

SF were seeded in 100-mm petri dishes ($3x10^5$cells/dish) and cultured in the absence or presence of 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa, for 24h. Total RNA was obtained using Tri®Reagent (Sigma-Aldrich). 2µg of RNA were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Semiquantitative real-time PCR (qPCR) analysis

was performed using a TaqMan® Gene Expression Master Mix with manufactured-predesigned primers and probes for β-actin (NM001101.3), ADAMTS-4 (NM005099.4), ADAMTS-5 (NM007038.3), ADAMTS-7 (NM014272.3) and ADAMTS-12 (NM030955.2) (Applied Biosystems). We normalized the target gene expression to the housekeeping gene, β-actin ($2^{-\Delta Ct}$). For relative quantification, results were presented as the relative expression with respect to the untreated condition using the formula $2^{-\Delta\Delta Ct}$, as previously described.[19]

Quantification of ADAMTS in culture supernatants

SF were seeded in 6-well plates ($6x10^4$cells/well) and cultured in the absence or presence of 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa, for 24h. Levels of ADAMTS were measured in the culture supernatants using commercial ELISA kits for ADAMTS-4 and -5 (Cloud-Clone Corp, Houston, Texas, USA), and for ADAMTS-7 and -12 (MyBioSource, San Diego, CA, USA).

Aggrecanase activity assay

SF were seeded in 100-mm petri dishes ($3x10^5$cells/dish) and cultured in the absence or presence of 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa, for 24h. Aggrecanase activity was measured in the SF culture supernatants using a Sensitive Aggrecanase Activity ELISA Kit (MD Bioproducts, Zürich, Switzerland), according to the manufacturer's instructions. Briefly, this assay consists in two modules. In the Aggrecanase Module, a modified interglobular domain (aggrecan-IGD-s) is digested with aggrecanases, and its proteolytic cleavage releases an aggrecan peptide (ARGSVIL-peptide-s), which is then quantified with antibodies in the ELISA Module.

Immunocytochemistry

SF were seeded on glass coverslips ($2.5x10^4$cells/glass), fixed with paraformaldehyde, and permeabilized with Tween-20 in phosphate buffered saline (PBS). Cells were blocked with PBS containing donkey serum and incubated with rabbit polyclonal anti-human antibodies for ADAMTS-4, -5, -7 or -12 (Sigma-Aldrich). After washing, cells were incubated with AlexaFluor 488 donkey anti-rabbit IgG antibody (Invitrogen). Coverslips were counterstained with Hoechst. Background fluorescence was reduced with Sudan Black in ethanol. Negative controls were performed in the absence of primary antibodies (data not shown). Fluorescence was examined using an Olympus BX51 microscope with DP72 camera model (objective 40X).

Runx2 assay

SF were seeded in 150-mm petri dishes ($8x10^5$cells/dish). A Nuclear Extract Kit (Active Motif, Rixensart, Belgium) was used for nuclear extracts preparation, and the protein content was measured with a QuantiPro™ BCA Assay Kit (QBCA) (Sigma-Aldrich). Cytoplasmic extracts obtained were stored at -80°C for later use in the Western blots. Nuclear extracts (12µg/well) were added to a 96-well plate and Runx2 activity was measured using a TransAM™ AML-3/Runx2 kit (Active Motif). Time-course of Runx2 activation after incubation with 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa was studied (data not shown), and the experiments were performed at 60 or 30 minutes of treatment, respectively.

β-catenin assay

To detect β-catenin levels, a β-catenin (Total) and a (Phospho) InstantOne™ ELISA kits were used (eBioscience, San Diego, CA, USA) with SF cellular lysates. Briefly, SF seeded in 100-mm petri dishes ($3x10^5$cells/dish) were scraped into PBS,

centrifuged and resuspended in the Cell Lysis Buffer Mix (eBioscience). Protein content was measured by QBCA. Levels of β-catenin in the cellular lysates were measured after 60 minutes of treatment with 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa.

Western blots

For the detection of ADAMTS, SF were seeded in 100-mm dishes and cultured to confluence. Culture supernatants were collected. For protein purification and concentration, Amicon Ultra 0.5 mL centrifugal filters (Merck Millipore, Darmstadt, Germany) were used. For Runx2 and β-catenin, the cytoplasmic extracts previously obtained were used, and protein content was measured by QBCA. Cytoplasmic extracts (15µg/well), and culture supernatants were subjected to SDS-PAGE and blotted on a PVDF membrane (Bio-Rad Laboratories, France).

Membranes were blocked with Tris-buffered saline containing BSA and Tween-20, and incubated with mouse monoclonal anti-human ADAMTS-4, ADAMTS-5 (R&D Systems), Runx2 or β-catenin (Santa Cruz Biotechnology) antibodies, or rabbit polyclonal anti-human ADAMTS-7 or ADAMTS-12 antibodies (Abcam, UK). Appropriate horseradish peroxidase-conjugated secondary antibodies were applied and detected by Western blot Luminol Reagent (Santa Cruz Biotechnology). For Runx2 and β-catenin, we used β-actin as a loading control. Protein bands were scanned and quantified with the Bio-Rad Quantity One program.

Blockade experiments

For blockade experiments, HD- and OA-SF were seeded in 100-mm dishes (3.10^5cells/dish) and cultured in serum-free DMEM with 1% L-glutamine and 1% antibiotic-antimycotic, in the absence or presence of 10 µM of MEK inhibitor, PD98059, consequently implicated in the inhibition of ERK-MAPK; 10 µM of p38-

MAPK inhibitor, SB203580 (Calbiochem, EMD Biosciences, San Diego, CA), or 200 ng/ml of Wnt inhibitor, DDK-1 (R&D Systems), for 1 hour. These treatments were followed by stimulation with 10 ng/ml IL-1β or 10 nM Fn-fs 45 kDa, for 24 hours. Total RNA was obtained and RT-qPCR for ADAMTS-4 and -5 was performed as previously described.

GAGs and COMP assays in cartilage-SF co-cultures

Release of GAG and COMP from cartilage was measured in culture supernatants from wells containing co-cultures of SF over cartilage explants[20]. OA human cartilages were obtained from 3 patients undergoing total hip arthroplasty from Hospital del Mar (Barcelona, Spain). Fixed diameter (6mm) and height (2mm) sections were collected from cartilage areas without macroscopically signs of OA. Samples were frozen at -80°C and stored until testing. 1 explant per well was attached to a 24 well plate. HD- or OA-SF were added drop-wise on top of the cartilage surface ($2x10^4$SF/explant). After 3 hours of incubation, wells were filled with DMEM in the absence or presence of 10ng/ml IL-1β or 10 nM Fn-fs 45 kDa, and cultures were continued for 14 days. Culture supernatants were collected for detection of GAG and COMP, using a Blyscan™ Sulfated Glycosaminoglycan Assay (Biocolor Ltd County Antrim, Ireland, UK), and a Quantikine® Human COMP Immunoassay (R&D Systems, Abingdon, OX, UK), respectively. Frozen sections were prepared using a cryostat and stained with Alcian blue and Callejas's tricromic. Sections were observed using an Olympus BX51 microscope with DP72 camera model (objective 20X).

Statistical analysis

Data were analyzed using the GraphPad Prism software (version 6). Data were subjected to normality test (Kolmogórov-Smirnov test) and equal variance test (F-test). Statistical differences between sample groups were assessed using Student's two-tailed *t*-test or unpaired *t*-test with Welch's correction, in case of groups with different variances. $P^{<}$ 0.05 were considered statistically significant. Results are presented as the means ± SEM.

Results

SF express and release ADAMTS-4, -5, -7 and -12 in HD and OA patients

We explored the constitutive expression of ADAMTS in HD- and OA-SF by RT-qPCR (Fig.1). Patterns of constitutive ADAMTS gene expression were similar in untreated HD- and OA-SF. ADAMTS-5 was the most expressed followed by ADAMTS-4, ADAMTS-7 and ADAMTS-12. ADAMTS-5 transcripts were nearly 50-fold higher than ADAMTS-12. Comparing HD- and OA-SF, ADAMTS-4 and -7 transcripts were 2 and 4-fold higher in OA- than in HD-SF, respectively. However, ADAMTS-5 and -12 mRNA levels were similar.

ADAMTS protein expression was confirmed by immunocytochemistry. Untreated HD- and OA-SF displayed similar morphology and both showed cytoplasmic immunostaining for ADAMTS-4, -5, -7 and -12 (Fig.2). No staining was observed in isotype controls (data not shown).

ADAMTS were also evaluated in untreated SF culture supernatants by Western blot (Fig.3). We confirmed that all ADAMTS are released to the medium by HD- and OA-SF. Western blots for ADAMTS-4 and -5 (Fig.3A,B) revealed bands corresponding to the active forms (between 48 and 74 kDa), and additional bands with a higher molecular weight. ADAMTS-7 and ADAMTS-12 Western blots (Fig.3C,D) showed

bands with the predicted molecular weight of the enzymes (between 114 and 201 kDa), and additional smaller bands.

IL-1β and 45kDa Fn-fs enhance the expression of ADAMTS-4 and ADAMTS-5 in SF

Since SF has been suggested to represent an important source of aggrecanases within the joint mediating cartilage destruction,[10] we next studied the effect of IL-1β and Fn-fs on their production. IL-1β and Fn-fs increased the transcript and protein of ADAMTS-4 in HD- and OA-SF compared with the untreated cells (Fig.4A,B). A significant increase in ADAMTS-5 transcript and protein was detected in OA-SF for both stimuli, while in HD stimulation was only observed in the protein after treatment with IL-1β (Fig.4C,D). Altogether, these results show that IL-1β increased ADAMTS-4 and -5 in HD- and OA-SF, while Fn-fs showed more specific effects in OA, resulting in a significant augment of ADAMTS-4 in both, and in a restricted stimulation of ADAMTS-5 production in OA-SF.

Aggrecanase activity in SF and GAGs release in cartilage-SF co-cultures

ADAMTS-4 and -5 cleave aggrecan within the interglobular domain at the Glu-373 and Ala-374 bond.[21, 22] Thus, we assessed the ability of ADAMTS-4 and -5 produced by cultured SF to this cleavage by measuring aggrecanase activation and the ARGSVL-peptide-s released in culture supernatants by means of ELISA.

Both, the constitutive aggrecanase activity (Fig.4E) and the derived peptide (data not shown) were significantly greater in OA- compared to HD-SF. Fn-fs significantly increased aggrecanase activity (Fig.4F) and the derived peptide (data not shown) exclusively in OA-SF, while IL-1β did not induce any change. Interestingly, these results correlated with the Fn-fs induction of ADAMTS-4 and -5 in OA-SF.

The aggrecanase activity yields the generation of GAGs from the aggrecan in cartilage ECM. Hence, we next studied the potential capacity of SF to degrade cartilage by measuring GAG release in supernatants from cartilage-SF co-cultures. After 14 days of *in vitro* co-cultures a monolayer of SF was observed exclusively on the cartilage surface (Fig.4G). The effects of IL-1β and Fn-fs after 14 days of treatment were evaluated. The constitutive release of GAGs to the medium was significantly greater in OA-SF compared to HD-SF. IL-1β induced no change in GAGs levels, whereas Fn-fs enhanced significantly the release of GAGs in both HD- and OA-SF (Fig.4I).

Runx2 and β-catenin activation in SF

Since Runx2 transcription factor and Wnt/β-catenin signaling are involved in aggrecanases gene expression,[16-18] and also seem to be implicated in the OA pathology,[23, 24] we decided to examine whether IL-1β or Fn-fs could alter the ADAMTS-4 and -5 expressions by the modulation of these factors.

Indeed, we found that both IL-1β and Fn-fs significantly induced nuclear activation of Runx2 in HD and OA-SF (Fig.5A), consistent with its reduction in the cytoplasm after the stimulation with both mediators (Fig.5C). Nonetheless, the data were consistent with a role for Runx2 in ADAMTS4 transcription since the transcript abundance with IL-1β, on both HD and OA, was about 2.0 fold greater than in untreated controls. Further, treatment with Fn-fs stimulated ADAMTS4 expression about 2.5 fold in OA cells and about 1.3 fold in HD cells. In contrast to ADAMTS4, ADAMTS5 expression showed no stimulation of HD-SF by either IL-1β or Fn-fs, but about a 1.6 fold increase by both IL-1β and Fn-fs in OA.

In the cytoplasm, β-catenin is regulated by interaction with a multiprotein complex that phosphorylates it to be degraded by proteasomes. Upon activation of Wnt signaling, non-phosphorylated β-catenin is transported to the nucleus, where it couples with the complex TCF/LEF (T-cell factor/lymphoid-enhancing factor) to initiate the transcription of ADAMTS-4 and -5 genes.[25] We measured both β-catenin forms in cellular lysates of SF, where levels of phosphorylated β-catenin were undetectable by ELISA (data not shown). Thus, we measured the total β-catenin that mainly represented the active form.

The β-catenin content of whole cell lysates was about 1.5 fold higher for untreated OA-SF than untreated HD. Moreover β-catenin levels increased to about 2 folds after treatment with either IL-1β or Fn-fs exclusively in OA (Fig.5B), which correlates with the reduction observed in cytosplasmic extracts by Western blot (Fig.5D).

To better elucidate the implication of Runx2 and β-catenin in the aggrecanases expression, we performed blockade experiments using inhibitors of two MAPK, ERK and p38-MAPK, implicated in the activation of Runx2, PD98059 and SB203580, respectively. We also used an inhibitor of Wnt/β-catenin signaling, DDK-1. We showed that PD98059 significantly inhibited mRNA expression of ADAMTS-4 before treatment with IL-1β or Fn-fs, in HD- and OA-SF (Fig.6A). Moreover, PD98059 inhibited the expression of ADAMTS-5 in OA-SF after both stimuli, while in HD this inhibition was observed only before stimulation with IL-1β (Fig.6B). On the other hand, SB203580 is involved in the decrease of ADAMTS-4 mRNA expression stimulated by Fn-fs in both, HD- and OA-SF (Fig.6A). Regarding ADAMTS-5, SB203580 significantly inhibited its expression only in OA-SF, after both stimuli (Fig.6B). Moreover, the Wnt/β-catenin inhibitor DKK-1 significantly inhibited the

expression of both, ADAMTS-4 and -5, before treatment with IL1β or Fn-fs, exclusively in OA-SF (Fig.6C,D).

Induction of ADAMTS-7 and ADAMTS-12 by IL-1β or 45kDa Fn-fs in SF, and COMP production in cartilage-SF co-cultures

We further studied the effects of IL-1β and Fn-fs on the ADAMTS involved in the degradation of COMP. ADAMTS-7 and -12 share a C-terminal COMP/GEP-binding TSP domain. Their effects in OA are due to the association of this domain with COMP and its subsequent degradation.[26] Significant increases in ADAMTS-7 transcript and protein were detected after IL-1β and Fn-fs stimulation (Fig.7A,B). Regarding ADAMTS-12, we observed an increase of mRNA and protein induced by both stimuli exclusively in OA-SF (Fig.7C,D).

The release of COMP and its degradative fragments was measured in the cultures supernatants after 14 days of treatment with IL-1β and Fn-fs (Fig.7E). The constitutive release of COMP was significantly greater in OA-SF than in HD. Moreover, significant increases were detected after IL-1β and Fn-fs stimulation.

Discussion

ADAMTS metalloproteinases play key roles in cartilage homeostasis and in the pathogenesis of OA, where the disruption of this balance, in favor of proteolysis, leads to a pathological cartilage destruction.[3] ADAMTS-4, -5, -7 and -12 have been implicated in the breakdown of cartilage in OA,[27, 28] ADAMTS-4 and -5 degrading aggrecan, and ADAMTS-7 and -12 degrading COMP. Although their functions are well understood in cartilage, few studies have addressed the contribution of SF to their expression and release.

We showed that ADAMTS-5 was the most expressed in both HD- and OA-SF. While ADAMTS-4 gene expression was higher in OA than in HD, ADAMTS-5 mRNA expression was similar in both. Despite levels of ADAMTS-5 are higher, differences in the ADAMTS-4 expression between HD and OA-SF, seems to indicate the involvement of this ADAMTS in the OA pathology. However, lower levels of ADAMTS-4 and -5 have been reported in OA cartilage and synovium compared with non-OA tissues.[29] Nevertheless, this discrepancy could be explained by the fact that whole synovium was used in their experiments. In addition, discordant data in the constitutive mRNA expression of ADAMTS-4 and -5 in non-OA and OA cartilage have been described, [10] likely due to the different stages of the disease.

The complexity of ADAMTS regulation, by both pre- and post-transcriptional mechanisms, which ultimately determine the levels of secreted enzymes, has been reported.[10] In this study we demonstrated that both HD- and OA-SF release aggrecanases. Western blot analysis of ADAMTS-4 and -5 in SF culture supernatants revealed the presence of the active forms of both enzymes, between 48 and 74 kDa, similar to those reported in OA synovial fluids.[30] We also observed a major band between 74 and 114 kDa, which has been described as the ADAMTS-4 proenzyme, in cartilage from OA patients,[28] as well as in SF.[9] Regarding ADAMTS-5, other authors have also reported a 70 kDa form of ADAMTS-5 in cartilage, as well as in SF from OA patients,[9, 28] that could represent a different degradation fragments.

We reported that the aggrecanase activity and GAGs release are constitutively greater in OA than in HD. By contrast, no differences in the expression of aggrecanases between non-OA and OA synovium have been reported,[9, 31] with no data about their activity. Our results showed that IL-1β increased ADAMTS-4 and -5 with no impact in the aggrecanase activity or GAGs production. Since the protein

abundance of ADAMTS-4 is apparently influenced by the transcript, in both HD and OA, whereas the protein abundance of ADAMTS-5 after stimulation with IL-1β in HD does not correlates with the transcript, it seems likely that this ADAMTS is not the main aggrecanase. This fact corroborates other studies, which indicate that ADAMTS-5 mRNA levels do not correlate with the OA progression in chondrocytes, due to post-translational regulations.[32] A recent study reported that IL-1β induced expression of ADAMTS-4 in SF but did not measure the activity. [33] At functional level, our results are in agreement with previous findings showing that ADAMTS-4 and -5 are not regulated by this cytokine in SF.[9, 12, 31] The role of IL-1β in OA is controversial and the implication of other mediators, as cartilage ECM degradation products, seems to be more relevant in the pathology.[12] Despite that, the study of IL-1β, as a pleiotropic pro-inflammatory cytokine, also contributes to the knowledge of the mechanism involved in the disease. In addition, since synovial inflammation intensity is greater in OA initial stages,[4] the lack of effects observed in the aggrecanase activity after IL-1β stimulation could be explained by the advanced disease state of our patients. Although, after stimulation with IL-1β we observed effects in the levels of ADAMTS-4 and -5, they were not significant enough to promote their activity.

Articular cartilage matrix proteins are degraded in OA resulting in the production of fragments with pro-inflammatory properties, including those of fibronectin.[34, 35] Whereas in chondrocytes it has been described the role of N-terminal Fn-fs 45kDa inducing matrix metalloproteinases synthesis and aggrecan degradation,[13, 14, 36] there are no data about its action in other joint cells. Here we describe for the first time the aggrecanases induction by Fn-fs in SF. In line with these previous reports, we noticed that Fn-fs produced a more specific effect in OA-SF increasing aggrecanases production and activity, as well as cartilage degradation, evaluated by GAGs release

from SF-cartilage co-cultures. Since ADAMTS-4 was also increased by Fn-fs in the co-cultures with HD- and OA-SF, these results again points to its main contribution to the aggrecan degradation. The integrin α5β1 is one of the main the receptors implicated in the function of fibronectin, being involved in the cartilage proteoglycan degradation induced by the 45 kDa Fn-fs.[37, 38] α5β1 integrins are also expressed in RA-SF showing a significant increase compared with normal SF.[39] Thus SF from OA patients would synthetize aggrecanases after 45kDa Fn-fs stimulation through integrins engagement. Our data suggest that, in stages of SF hyperplasia in OA, Fn-fs could establish a feedback loop contributing to the maintenance of cartilage erosion.

Runx2 can be predicted to promote ADAMTS-4 and -5 transcription.[16, 17] A recent studies indicates that this transcription factor is also implicated in the expression of ADAMTS-7 and -12 in OA cartilage.[24] On the other hand, Wnt/β-catenin signaling is a potent stimulator of chondrocyte matrix catabolic action triggering joint destruction, which also regulates aggrecanases expression.[18] Both signaling pathways seem to play important roles in the OA pathophysiology.[40-43] We showed that IL-1β and Fn-fs induced Runx2 in HD- and OA-SF. Moreover, ERK- and p38- MAPK are implicated in the activation of Runx2 transcription factor.[44-46] Blockade experiments showed that ERK-MAPK is involved in the expression of ADAMTS-4 and -5, stimulated by IL-1β or Fn-fs, in OA-SF. As both stimuli also induced the activation of Runx2 and the expression of both aggrecanases in these cells, we can conclude that ERK-MAPK signaling through Runx2 is involved in the expression of ADAMTS-4 and -5 in OA. ERK-MAPK also regulates the expression of ADAMTS-4 after both stimuli in HD-SF, in correlation with the induction of ADAMTS-4 transcript and protein, as well as with the activation of Runx2. These data suggest that IL-1β and Fn-fs could control

ADAMTS4 transcription via ERK-MAPK and Runx2 also in SF from HD, by contrast to ADAMTS5. On the other hand, p38-MAPK is only implicated in the expression of ADAMTS-4 after Fn-fs induction in HD- and OA-FLS, as well as in the induction of ADAMTS-5 exclusively in OA-SF by both stimuli.

Of interest, we detected β-catenin induction exclusively in OA-SF, which also correlates with the restricted inhibition of Wnt signaling in OA-SF, mediated by DKK-1 before stimulation by both, IL-1β or 45kDa Fn-fs, pointing to the implication of this signaling pathway in the OA pathology. These results are in agreement with the higher expression of Wnt responsive genes, such as WISP-1, in OA,[47] which has been associated with a profibrotic and antichondrogenic OA-like phenotype.[50] This interpretation is further supported by the finding that the Wnt7a/β-catenin pathway promotes proteasomal degradation of Sox9, thereby blocking expression of chondrogenic genes.[48]

These results could shed some light on the understanding of how synovitis is triggered in OA, which is an issue under exploration. The disruption of the articular cartilage matrix is the most differencing feature in OA, and the resulting fragments of ECM catabolism have been associated to inflammation through the triggering of Toll-like receptors (TLRs) signaling pathways. Both, IL-1β and Fn-fs induced the expression of TLR2 in chondrocytes.[49] Thus, in addition to integrins, Fn-fs, as an endogenous ligand of TLR2 and TLR4, could act through its engagement, given that both receptors are present in SF.[50, 51] Moreover, both stimuli induce the activation of MAPK signaling, implicated in the activation of Runx2 and other transcription factors as the nuclear factor-κB (NF-κB), [13-15, 46, 52] which also induce the expression of Wnt. [53, 54] Therefore, we can hypothesize that IL-1β and 45 kDa Fn-fs induce aggrecanases expression by activation of the transcription factor Runx2, mainly

through the ERK-MAPK signaling. Moreover, these stimuli specifically induce the Wnt/β-catenin signaling in OA-SF, with the consequent therapeutic value.

ADAMTS-7 and -12 have been detected mainly in cartilage, with an upregulated expression of their transcripts in OA compared with HD. Regarding other joint tissues, ADAMTS-7 and -12 mRNA expression have been previously described in whole synovium, with similar levels in OA and HD.[29] In the present study we show for the first time, that isolated SF express and release ADAMTS-7 and -12. ADAMTS-7 trancripts were higher in OA- than in HD-SF, while expression of ADAMTS-12 was similar in both. The higher expression of ADAMTS-7 in OA compared with HD also indicates the contribution of this ADAMTS in the OA pathology.

Our results are the first to describe the presence of ADAMTS-7 and -12 proteins in SF. Western blot of ADAMTS-7 showed a band between 114 and 201 kDa, similar to the active form previously described in a human kidney cell line.[55, 56] Regarding ADAMTS-12, we also reported a band between 114 and 201 kDa, equivalent to the previously reported in the same human line,[33] and a smaller band between 74 and 114 kDa that could represent the C-terminal fragment containing the TSP-1 repeats.[57, 58]

As ADAMTS-7 and -12 are involved in the breakdown of arthritic articular cartilage,[56, 59] SF represent other source of both metalloproteinases that would contribute to the maintenance of the cartilage damage. In this sense, COMP fragments have been identified in cartilage, synovial fluid and serum from OA and rheumatoid arthritis patients. Moreover, increased levels of COMP in synovial fluid and serum are related to joint damage and progression in rheumatic diseases.[59-61] Different joint tissues such as bone, cartilage, synovium and tendon contain and express ADAMTS-7,

which co-localizes with ADAMTS-12 and COMP in the cytoplasm of chondrocytes.[62, 63] In line with this, we reported here that cartilage-OA-SF co-cultures constitutively release COMP to the medium at significant higher levels than cartilage-HD-SF co-cultures.

TNFα and IL-1β increase ADAMTS-7 and -12 mRNA in cartilage explants,[64] whereas ADAMTS-12 is not induced in human fetal fibroblasts.[57] We report, for the first time in SF, that IL-1β and Fn-fs promoted significant increases in ADAMTS-7 transcripts and protein levels, being greater in OA- than in HD-SF. Similarly, ADAMTS-12 transcripts and protein were stimulated by IL-1β and Fn-fs specifically in OA-SF. Interestingly, ADAMTS-12 was the only ADAMTS studied exclusively induced in OA-SF. Those facts are important for the development of combined therapies based on the blockade of these ADAMTS. Emergent data indicate that ADAMTS-12 has multiple functions in the inflammatory response,[64] angiogenesis, and apoptosis.[65-67] Thus, further studies are needed to clarify whether ADAMTS-12 is also involved in those functions during OA development.

Our results showed that the stimulation of COMP release by IL-1β and Fn-fs was greater in OA-SF. Since specific antibodies against ADAMTS-7 and -12 inhibited the TNFα- or IL-1β-induced COMP degradation in the cartilage of OA patients,[68] this increased release of COMP after IL-1β or Fn-fs treatment could be ascribed to the increased expression of both ADAMTS. Since, by contrast to ADAMTS-12, IL-1β and Fn-fs also induced ADAMTS-7 in HD-SF, COMP degradation may be mainly attributed to its action. Our data suggest that, in the late phase of OA, IL-1β and Fn-fs may contribute to the damage of non-collagenous components of the ECM by increasing ADAMTS-7 and -12.

Our study presents two potential limitations. The *in vitro* model of cartilage-SF co-culture uses dead cartilage, thus whether the same results would be obtained with SF adhered to live human OA cartilage is unknown. Besides, the medium used for all cell cultures was DMEM containing high glucose concentration. However, given that all treatments were performed with the same medium, this condition would not invalidate our results.

Overall, our data indicate that SF provide aggrecanases, ADAMTS-7, and ADAMTS-12 that contribute to the chronicity and destruction of OA joint. While both IL-1β and Fn-fs have been described as mediators of cartilage degradation in OA, our findings indicate that, despite the contribution of both mediators, they are Fn-fs rather than IL-1β, which plays the major pathologic role, in agreement with recent studies.[12] We showed that constitutive levels of ADAMTS-4, one of the main aggrecanases in cartilage destruction, were higher in OA than in HD. Interestingly, the higher levels of ADAMTS-7 in OA compared to HD, as well as the regulation of ADAMTS-12 by IL-1β and Fn-fs exclusively in SF from OA patients, suggest their potential as new therapeutic targets for the treatment of the disease. Altogether, our results point to an important contribution of SF, providing the biochemical tools, to the chronicity and destruction of the osteoarthritic affected joint.

Acknowledgements

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Figure Legends

Figure 1. mRNA expression of ADAMTS in HD- and OA-SF. ADAMTS-4, -5, -7 and -12 mRNA expression was measured in untreated HD- and OA-FLS by RT-qPCR and normalized to β-actin using the formula $2^{-\Delta Ct}$ (see Patients and Methods). Values are presented as mean ± SEM of triplicate determinations (HD, n=4; OA, n=11). $^{\dagger}p<0.05$ and $^{\dagger\dagger\dagger}p<0.001$ HD vs OA.

Figure 2. Immunocytochemistry of ADAMTS in HD- and OA-SF. Protein expression of ADAMTS-4 **(A,B)**, ADAMTS-5 **(C,D)**, ADAMTS-7 **(E,F)**, and ADAMTS-12 **(G,H)** in untreated HD- and OA-FLS, was analyzed by immunofluorescence staining (green). Nuclei were counterstained with Hoeschst (blue). Figure shows immunostaining of the SF from one HD **(A,C,E,G)** and one OA patients **(B,D,F,H)**, representative of three independent experiments. Scale bars: 50µm. Fluorescence was examined on an Olympus BX51 microscope with DP72 camera mode (Objetive 40x).

Figure 3. Western blot of ADAMTS in HD- and OA-SF. Presence of ADAMTS-4 **(A)**, ADAMTS-5 **(B)**, ADAMTS-7 **(C)**, and ADAMTS-12 **(D)** was detected by Western blot in untreated HD- and OA-SF culture supernatants (HD, n=3; OA, n=3). MW markers on the PVDF membrane are shown.

Figure 4. Induction of ADAMTS-4 and -5 by IL-1β or 45kDa Fn-fs in HD- and OA-SF. A, C. mRNA expression of ADAMTS-4 and -5 after treatment with IL-1β or 45kDa Fn-fs for 24h, was measured by RT-qPCR, normalized to β-actin and presented as the relative quantification with respect to the untreated cells using the formula $2^{-\Delta\Delta Ct}$ (see Patients and Methods). Values are presented as mean ± SEM of triplicate determinations (HD, n=4; OA, n=7). **B, D.** Presence of ADAMTS-4 and -5 after

treatment with IL-1β or 45kDa Fn-fs for 24h, was determined by ELISA in culture supernatants. Concentrations were calculated based on the standard curve provided. Values are presented as the percentage of untreated cells (mean ± SEM of duplicate determinations) (HD, n=4; OA, n=7). **E, F.** Aggrecanase activity in culture supernatants was measured by an Aggrecanase Activity ELISA kit, in untreated cells **(E)**, and after treatment with IL-1β or 45kDa Fn-fs for 24h, presented as the percentage of untreated cells **(F).** Values are presented as mean ± SEM of duplicate determinations (HD, n=4; OA n=5). **G, H.** Representative histological sections of a cartilage explant (asterisk) in co-culture with OA-SF (arrow) **(G)**, and without SF **(H)**. Alcian blue and Callejas's tricromic staining (objective 20X). **I.** GAGs in cartilage-SF co-cultures supernatants after treatment with IL-1β or 45kDa Fn-fs for 14 days, were detected by a Blyscan™ Sulfated Glycosaminoglycan Assay. Values are presented as mean ± SEM of duplicate determinations (HD, n=3; OA n=3). Dashed lines represent the untreated condition. $^{\dagger}p<0.05$ HD vs OA; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ treatment vs untreated.

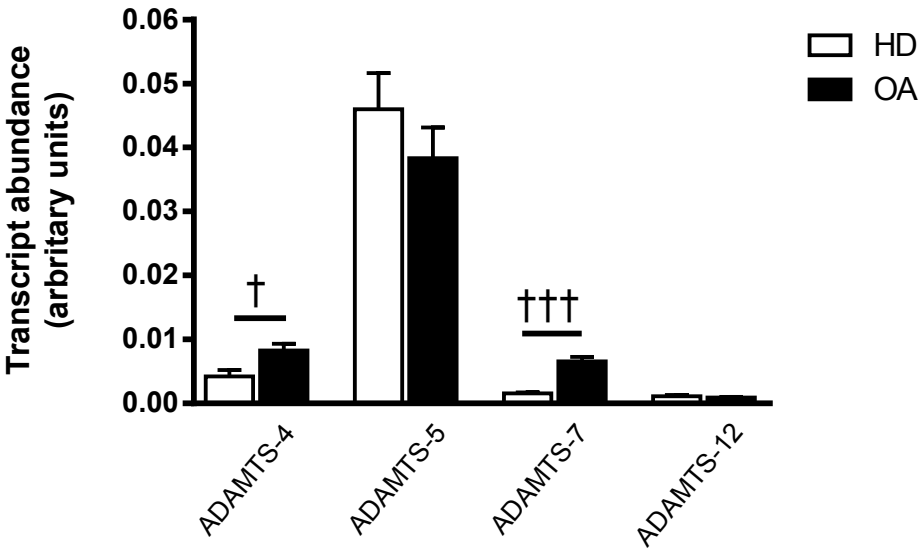
Figure 5. Activation of Runx2 and β-catenin by IL-1β or 45kDa Fn-fs in HD- and OA-SF. A. Runx2 activation was measured in nuclear extracts after 60 minutes of treatment with IL-1β or 30 minutes of treatment with 45kDa Fn-fs, using a TransAM™ AML-3/Runx2 kit. Values are presented as mean ± SEM of duplicate determinations (HD, n=4; OA, n=4). **B.** β-catenin was detected in cellular lysates after 24h of treatment after 60 minutes of treatment with IL-1β or 45kDa Fn-fs, by ELISA. Values are presented as mean ± SEM of duplicate determinations (HD, n=4; OA, n=5). **C, D.** Representative images of three independent experiments of the Western blots for Runx2 **(C)** and β-catenin **(D)** in cytoplasmic extracts, are shown. Protein bands were scanned and quantified with the Bio-Rad Quantity One program and presented.

Values are presented as the ratio of mean value intensity normalized to β-actin ± SEM of three independent experiments (HD, n=3; OA, n=3). †††p<0.001 HD vs OA; *p<0.05 treatment vs untreated.

Figure 6. Blockade of Runx2 and Wnt/β-catenin signaling. mRNA expression of ADAMTS-4 **(A, C)** and -5 **(B, D)** was measured by RT-qPCR, normalized to β-actin using the formula $2^{-\Delta Ct}$, and presented as the percentage of stimulated cells, after 1h of treatment with inhibitors of two MAPK implicated in the activation of Runx2, PD98059, an specific inhibitor of MEK, responsible of the activation of ERK-MAPK, and an inhibitor of p38-MAPK, SB203580 **(A, B)**, or an inhibitor of Wnt signaling, DDK-1 **(C,D)**, followed by treatment with IL-1β or 45kDa Fn-fs for 24h. Values are presented as mean ± SEM of triplicate determinations (HD, n=3; OA, n=3). *p<0.05, **p<0.01 and ***p<0.001 inhibition vs stimulation.

Figure 7. Induction of ADAMTS-7 and -12 by Fn-fs and IL-1β in HD- and OA-SF. **A, C.** mRNA expression of ADAMTS-7 and -12 after treatment with IL-1β or 45kDa Fn-fs for 24h, was measured by RT-qPCR, normalized to β-actin and presented as the relative quantification with respect to the untreated cells using the formula $2^{-\Delta\Delta Ct}$ (see Patients and Methods). Values are presented as mean ± SEM of triplicate determinations (HD, n=4; OA, n=7). **B, D.** Presence of ADAMTS-7 and -12 treatment with IL-1β or 45kDa Fn-fs for 24h, was determined by ELISA in culture supernatants. Values are presented as the percentage of untreated cells (mean ± SEM of duplicate determinations) (HD, n=4; OA, n=7). **E.** COMP in cartilage-SF co-cultures supernatants after treatment with IL-1β or 45kDa Fn-fs for 14 days, was detected by a Quantikine® Human COMP Immunoassay. Values are presented as mean ± SEM of triplicate determinations (HD, n=3; OA n=3). Dashed lines represent the untreated

condition. †p<0.05 HD vs OA; *p<0.05, **p<0.01 and ***p<0.001 treatment vs untreated.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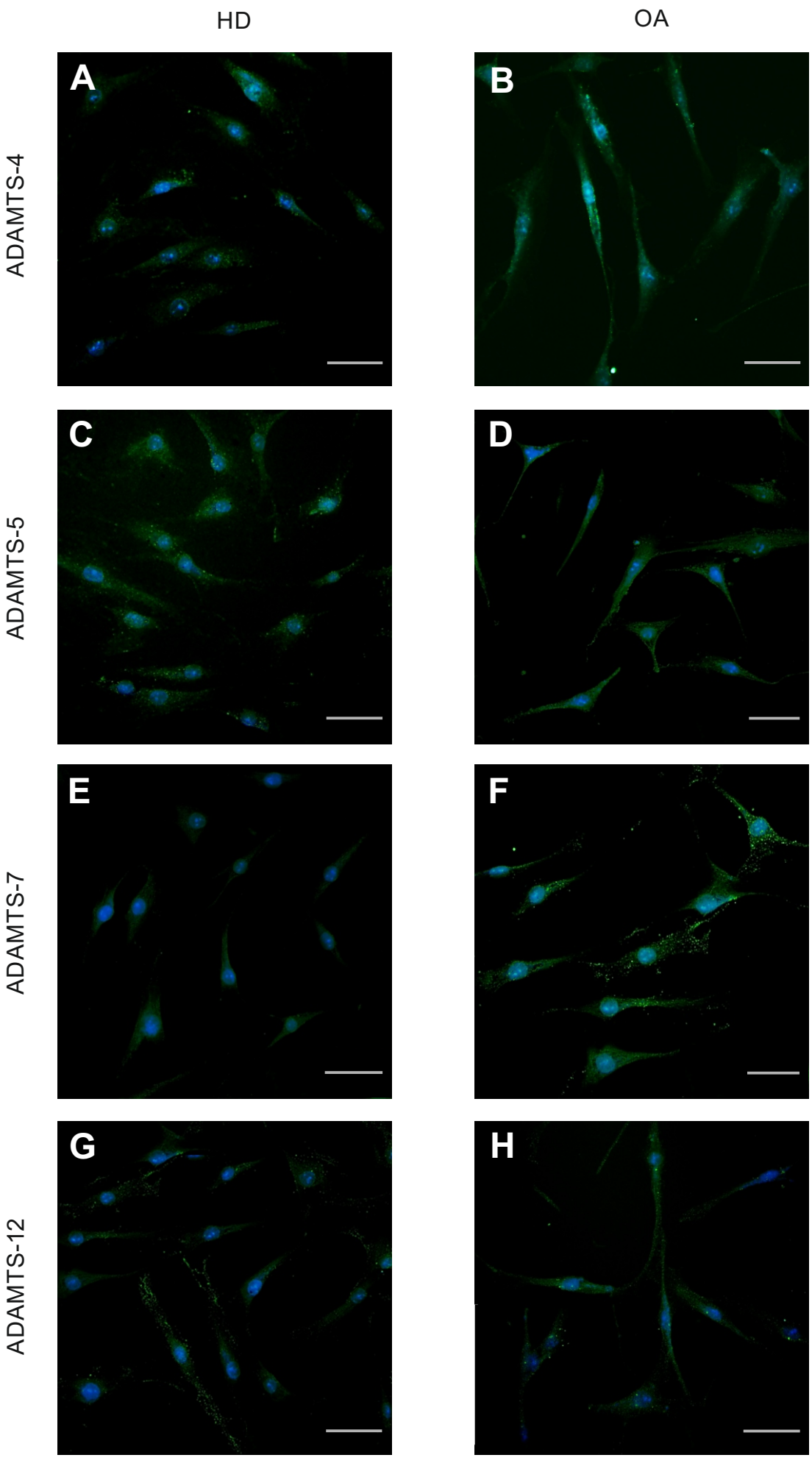
ADAMTS-12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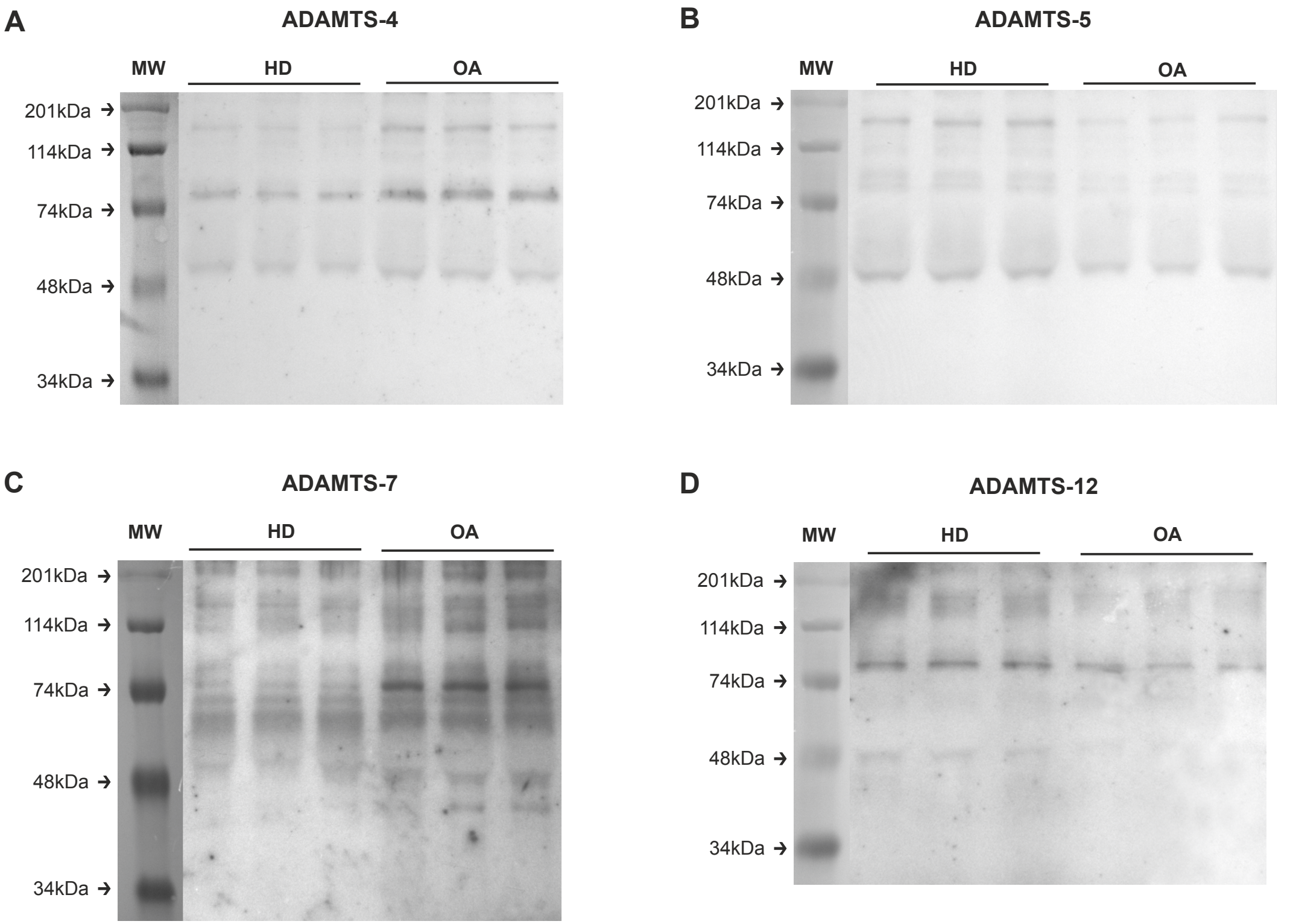
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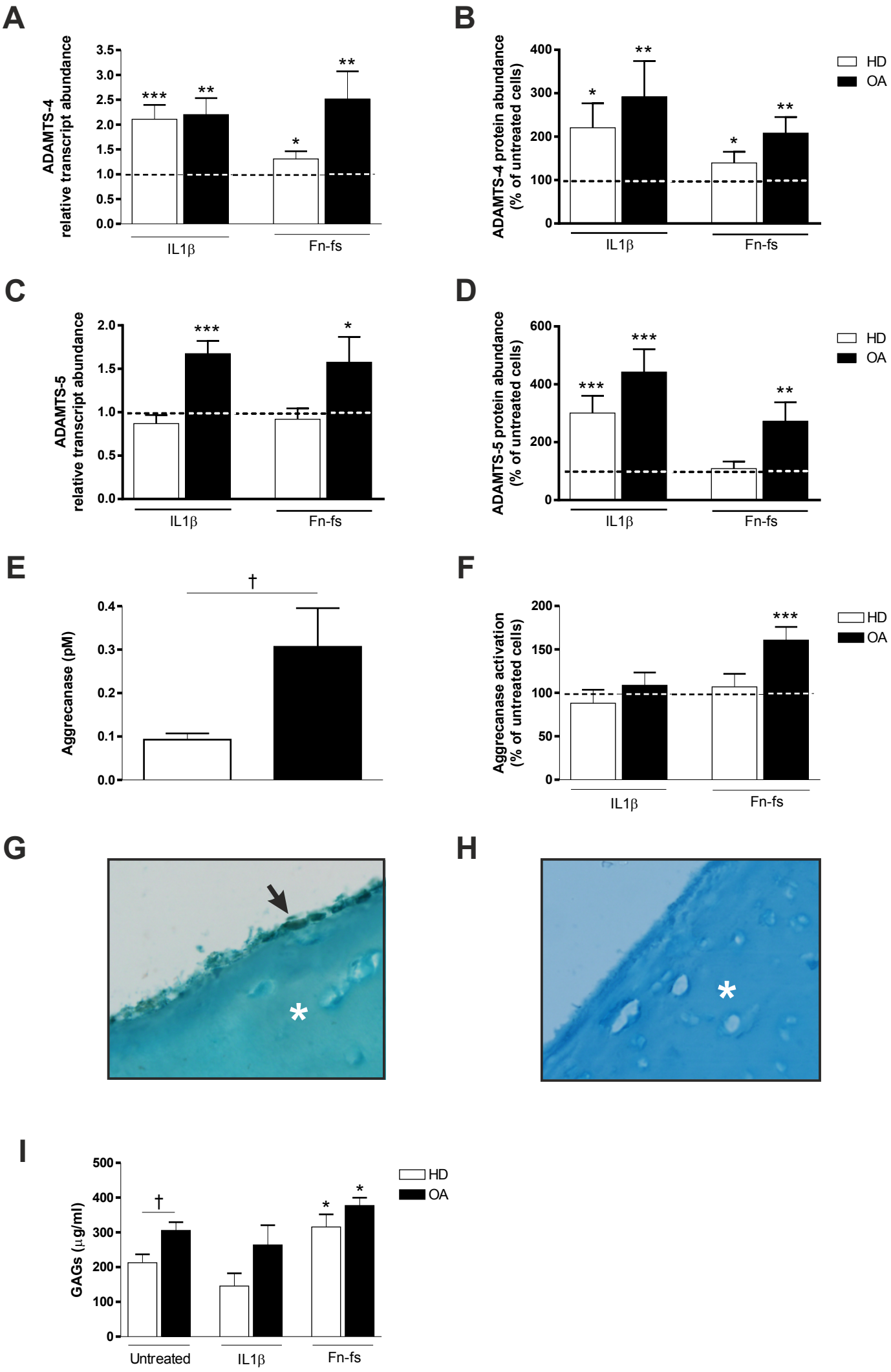
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ADAMTS-4
relative transcript abundance
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3.0
2.5
2.0
1.5
1.0
0.5
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**
*
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IL1β
Fn-fs
B
ADAMTS-4 protein abundance
(% of untreated cells)
400
300
200
100
0
*
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IL1β
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2.0
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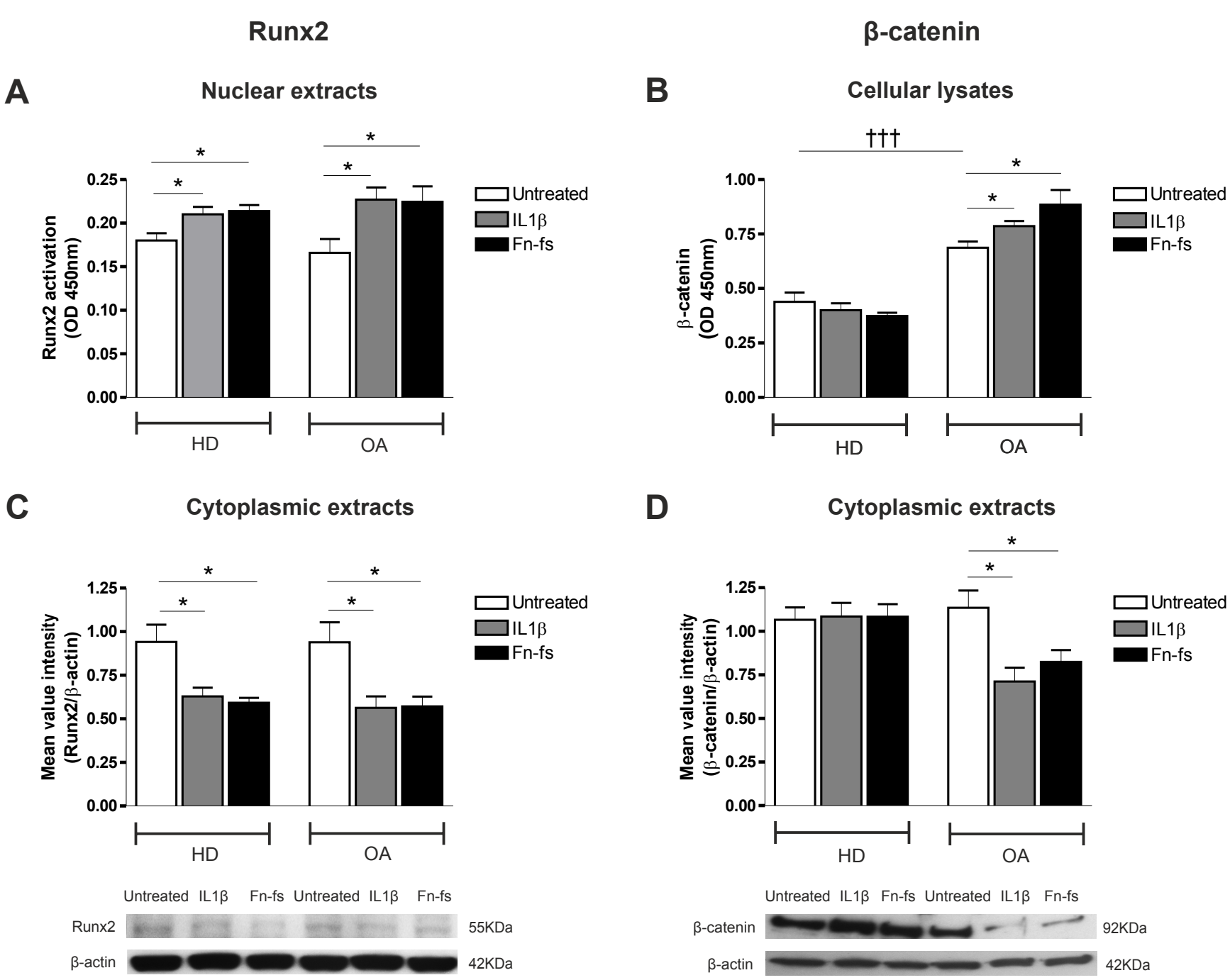
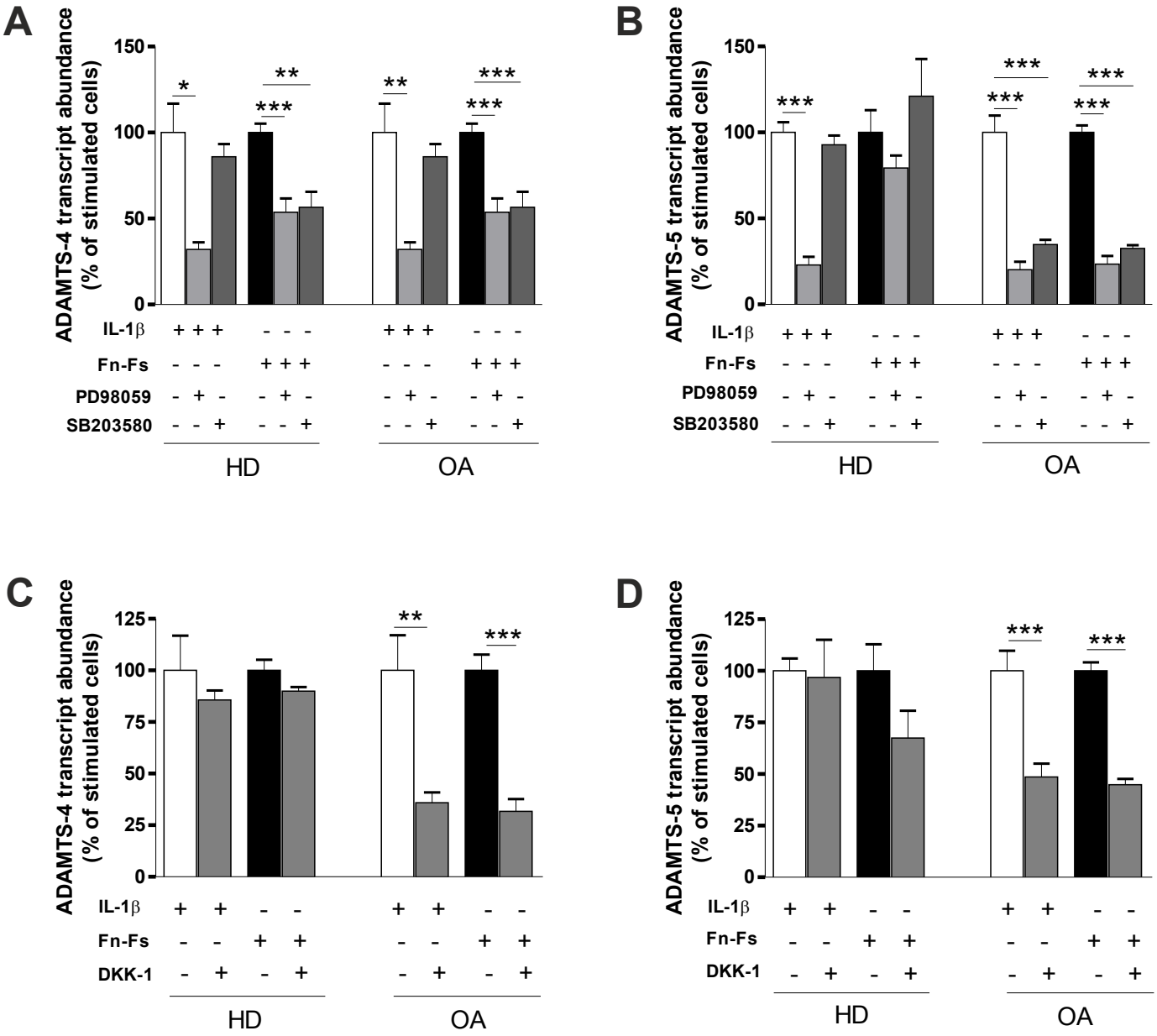
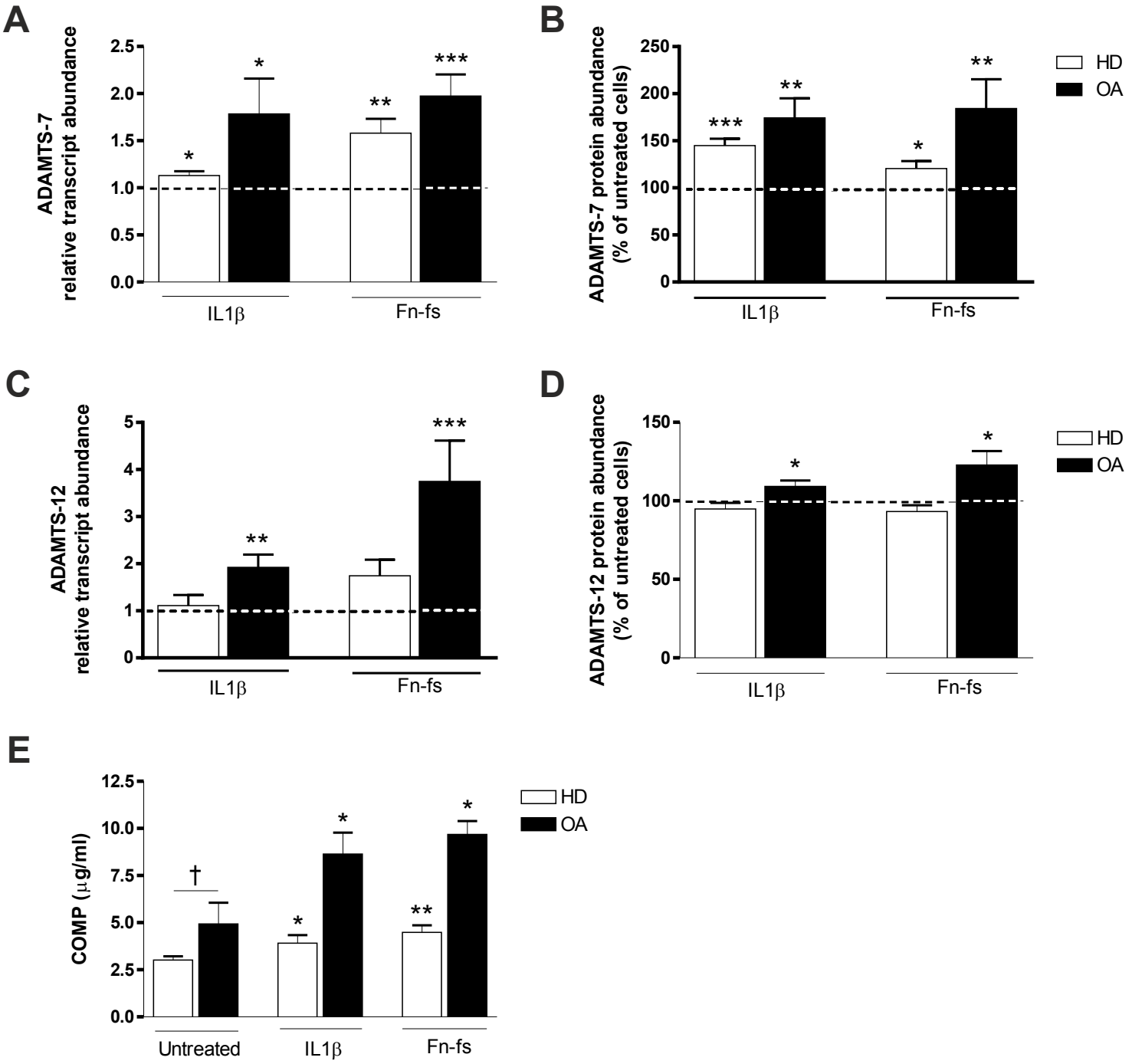
OA
E
Aggrecanase (pM)
0.4
0.3
0.2
0.1
0.0
†
F
Aggrecanase activation
(% of untreated cells)
200
150
100
50
0
***
IL1β
Fn-fs
HD
OA
G
*
H
*
I
GAGs (μg/ml)
500
400
300
200
100
0
†
*
*
Untreated
IL1β
Fn-fs
HD
OA


Runx2
β-catenin
A
Nuclear extracts
Runx2 activation (OD 450nm)
0.25
0.20
0.15
0.10
0.05
0.00
HD
OA
Untreated
IL1β
Fn-fs
B
Cellular lysates
†††
β-catenin (OD 450nm)
1.00
0.75
0.50
0.25
0.00
C
Cytoplasmic extracts
Mean value intensity (Runx2/β-actin)
1.25
Untreated IL1β Fn-fs Untreated IL1β Fn-fs
Runx2
55KDa
β-actin
42KDa
D
Cytoplasmic extracts
Mean value intensity (β-catenin/β-actin)
β-catenin
92KDa


A
ADAMTS-4 transcript abundance (% of stimulated cells)
150
100
50
0
*
**
***
**
***
IL-1β + + + - - - + + + - - -
Fn-Fs - - - + + + - - - + + +
PD98059 - + - - + - - + - - + -
SB203580 - - + - - + - - + - - +
HD
OA
B
ADAMTS-5 transcript abundance (% of stimulated cells)
150
100
50
0
***
***
***
***
***
IL-1β + + + - - - + + + - - -
Fn-Fs - - - + + + - - - + + +
PD98059 - + - - + - - + - - + -
SB203580 - - + - - + - - + - - +
HD
OA
C
ADAMTS-4 transcript abundance (% of stimulated cells)
125
100
75
50
25
0
**
***
IL-1β + + - - + + - -
Fn-Fs - - + + - - + +
DKK-1 - + - + - + - +
HD
OA
D
ADAMTS-5 transcript abundance (% of stimulated cells)
125
100
75
50
25
0
***
***
IL-1β + + - - + + - -
Fn-Fs - - + + - - + +
DKK-1 - + - + - + - +
HD
OA


A
ADAMTS-7
relative transcript abundance
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
***
IL1β
Fn-fs
B
ADAMTS-7 protein abundance
(% of untreated cells)
250
200
150
100
50
0
***
**
*
**
IL1β
Fn-fs
HD
OA
C
ADAMTS-12
relative transcript abundance
5
4
3
2
1
0
**
***
IL1β
Fn-fs
D
ADAMTS-12 protein abundance
(% of untreated cells)
150
100
50
0
*
*
IL1β
Fn-fs
HD
OA
E
COMP (μg/ml)
12.5
10.0
7.5
5.0
2.5
0.0
†
*
*
**
*
Untreated
IL1β
Fn-fs
HD
OA