Migfilin's elimination from osteoarthritic chondrocytes further promotes the osteoarthritic phenotype via β-catenin upregulation

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ABSTRACT

Osteoarthritis (OA) is a debilitating disease of the joints characterized by cartilage degradation but to date there is no available pharmacological treatment to inhibit disease progression neither is there any available biomarker to predict its development. In the present study, we examined the expression level and possible involvement of novel cell–ECM adhesion-related molecules such as Iintegrin Linked Kinase (ILK), PINCH, parvin, Mig-2 and Migfilin in OA pathogenesis using primary human articular chondrocytes from healthy individuals and OA patients. Our findings show that only ILK and Migfilin were upregulated in OA compared to the normal chondrocytes. Interestingly, Migfilin silencing in OA chondrocytes rather exacerbated than ameliorated the osteoarthritic phenotype, as it resulted in even higher levels of catabolic and hypertrophic markers while at the same time induced reduction in ECM molecules such as aggrecan. Furthermore, we also provide a link between Migfilin and β-catenin activation in OA chondrocytes, showing Migfilin to be inversely correlated with β-catenin. Thus, the present study emphasizes for the first time to our knowledge the role of Migfilin in OA and highlights the importance of cell–ECM adhesion proteins in OA pathogenesis.

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1. Introduction

Osteoarthritis (OA), a chronic degenerative disease of the joints, is the most common form of arthritis in older adults and a major cause of morbidity, activity limitation, physical disability, pain and reduction in quality of life. OA etiology is practically unknown although it is believed to be multifactorial. Mechanical load, joint injuries, obesity [1], and genetic predisposition are among the most serious risk factors for disease development. It is estimated that annually 15 million people are diagnosed with OA in the European Union while in the United States estimates indicate a rise in OA prevalence to 27 million in 2008 [1,2]. Although analgesics are used, to date there is no available pharmacological treatment to inhibit or reverse disease progression and thus, most patients end up undergoing joint replacement surgery. Moreover, to date

there is no available biomarker to predict OA development, leaving room for research in the field towards early diagnosis.

OA is generally characterized by progressive degeneration of the articular cartilage, which is accompanied by synovitis, formation of subchondral cysts, subchondral bone attrition, formation of osteophytes [3] and decrease in the content of extracellular matrix (ECM) proteoglycans leading to loss of cartilage elasticity and reduction of its ability to withstand mechanical load resulting in pain and difficulty during movement. Articular cartilage in healthy adults consists of chondrocytes with low metabolic activity [4] that do not undergo maturation, proliferation or apoptosis. In OA, however, the affected cartilage cannot maintain these characteristics and chondrocytes lose their differentiation, start expressing collagen 10A1 instead of collagen 2A1, enter the process of maturation, and acquire the ability to propagate [5] thus becoming hypertrophic cells [6]. Furthermore, to repair damage in the articular cartilage, osteoarthritic chondrocytes exhibit increased anabolic activity composing ECM components. However, this increased anabolic activity cannot compensate for the degradation taking place at the same time. Thus, osteoarthritic chondrocytes demonstrate at the same time dramatically elevated levels of catabolic enzymes such as matrix metalloproteases (MMP) and MMP-13 in particular

[5], and significantly reduced levels of ECM proteins such as aggrecan.

Although OA is considered to be a disease that affects the entire joint, the articular cartilage [7] remains the best studied component of the joint. Therefore, the investigation of osteoarthritic chondrocytes' characteristics is paramount. Considering the fact that chondrocytes are practically embedded in ECM, cell–ECM communication is of utmost significance for their homeostasis. This communication is achieved via a network of protein complexes with structural and functional role in transmitting signals [8], such as integrins and their binding partners. Thus, many cell–ECM adhesion proteins play important role in chondrocyte biology, as it has been shown that integrin alpha1 knock-out mice leads to OA [9], and β1 integrin knock-out in mice results in decreased chondrocyte proliferation [10]. In addition, Integrin-Linked Kinase (ILK) conditional knock-out in chondrocytes also causes decreased chondrocyte proliferation, impaired adhesion to ECM and chondrodysplasia [11]. Thus, inactivation or knock-out of integrin-associated genes and the ECM, leads to reduced chondrocyte proliferation and associated disorders, such as OA. Further evidence showing the importance of ECM/integrin-associated proteins in chondrocytes is the fact that ILK and its binding partners play an important role in the development and growth of tumors of chondrogenic origin [12].

It has been shown that when activated [13], ILK regulates three major survival signaling pathways; protein kinase B/Akt [13,14], Extracellular Signal-Regulated Kinase (Erk) [13], and Glycogen Synthase Kinase 3-β (GSK3β) pathway [13,15]. In fact, inactivation of GSK3β by ILK leads to induction of the transcription complex β-catenin/Lymphoid Enhancer Factor-1 (LEF1) [16], which in turn leads to increased regulation of multiple pro-survival genes. It should be noted that ILK forms a stable ternary complex with two other cell–ECM adhesion proteins, namely PINCH and α-parvin [17–20], which has been shown to be involved in cell shape regulation, migration, proliferation, and ECM synthesis [21]. Interestingly, however, ILK has been also shown to interact with multiple other novel ECM-adhesion related proteins including Mitogen inducible gene-2 (Mig-2, which is also known as kindlin-2) [22], which in turn, binds to Migfilin (also known as FBLP-1, filamin-binding LIM protein 1), a novel interactor of filamin [23] confirming the association between cell–ECM adhesion proteins and actin cytoskeleton.

Interestingly, similar type of proteins related to cellular organization, actin cytoskeleton and adhesion to the ECM have been identified by proteomic analysis to be expressed in chondrocytes [24,25] with elevated expression levels in OA patients compared to controls [26–28]. Nevertheless, none of the above-described ILK-related proteins has been studied in chondrocytes and their role in OA is completely unknown.

In the present study, to add on the association between ECM/integrin-associated proteins, we investigated, for the first time to our knowledge, the expression levels and possible involvement of ILK, PINCH, parvin, Mig-2 and Migfilin in OA pathogenesis.

2. Materials and methods

2.1. Articular cartilage samples

OA articular cartilage samples were obtained from femoral heads, femoral condyles and tibial plateaus of patients with primary OA undergoing knee replacement surgery at the Orthopaedics Department of University Hospital of Larissa. A total of 18 patients were included in the study. Each sample was categorized according to its gross morphology, as either severely damaged (OA = max), which was taken from the main defective area of maximal load, or mildly affected (OA = min) taken from areas with no obvious surface defects. Macroscopic findings were validated by histological studies and graded using the Mankin score (Mankin score for mild OA: 1–4 and for severe OA: 10–14). Radiographs were obtained before surgery and graded using the Kellgren–Lawrence system [29]. All patients had a Kellgren–Lawrence score >2. Patients with rheumatoid arthritis and other autoimmune diseases as well as chondrodysplasias, infection-induced OA and post-traumatic OA were excluded from the study.

Normal cartilage was obtained from 10 individuals with 0 Mankin score, undergoing fracture repair surgery, with no history of joint disease and no clinical manifestations of OA when specifically explored by radiographs.

Cartilage samples from both patients and healthy individuals were obtained following verbal informed consent. The method for obtaining verbal consent was approved by the Institutional Review Board of the University Hospital of Larissa. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Local Ethical Committee of the University Hospital of Larissa.

2.2. Primary chondrocyte isolation from articular cartilage

Primary chondrocytes were isolated from articular cartilage as described previously [29]. In brief, articular cartilage was dissected and subjected to digestion with 1 mg/ml pronase followed by overnight digestion with 1 mg/ml collagenase P (Roche). Chondrocytes were counted and checked for viability using trypan blue staining. Chondrocytes were cultured in Dulbecco's Modified Eagles Medium/Ham's F-12 (DMEM/F-12) supplemented with 5% fetal bovine serum and 1% penicillin–streptomycin for only 2 passages, to avoid dedifferentiation events occurring over time in culture.

2.3. RNA isolation and real time PCR

Total cellular RNA was extracted from cultured chondrocytes using Trizol reagent (Invitrogen). Transcription of 1 mg RNA to complementary DNA (cDNA) was performed using Superscript III (Invitrogen). Quantification of ILK, PINCH, α-parvin, mig-2, Migfilin, β-catenin, MMP-13, aggrecan, collagen 10A1 and collagen 2A1 expression was performed by real-time PCR (MiniOpticon, BioRad). Actin was used as housekeeping gene for α-parvin mRNA quantification while porphobilinogen deaminase (PBGD) was used as a housekeeping gene for all the other genes. Reactions were done in triplicate and at least 3 independent experiments were performed. All primers used are shown in Table 1. To quantify the relative expression of each gene, Ct values were normalized against the endogenous reference ($\Delta Ct = Ct$ target – Ct PBGD) and were compared with a calibrator using the $\Delta\Delta Ct$ method.

2.4. Protein extraction

Normal and osteoarthritic chondrocytes were lysed using RIPA lysis buffer containing 10 mM Tris(pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% Sodium Deoxycholated, 0.1% SDS, 1 mM EDTA, and a cocktail of protease and phosphatase inhibitors. Protein concentration was quantified using the BCA method (PIERCE).

2.5. Western blot analysis

Cell lysates from normal and OA chondrocytes were run in 10% acrylamide gels and transferred to PVDF membranes (Millipore). Signals were detected using suitable horseradish peroxidase-conjugated immunoglobulin. Anti-β-actin antibody (Sigma–Aldrich) was used as loading control. When necessary, Western blot bands from several different blots were quantified using the NIH Scion Image Software according to the guidelines.

Table 1
Primer sequences for real time PCR analysis.

Target gene	Sequence
ILK	Forward 5′ GAC ATG ACT GCC CGA ATT AG 3′
	Reverse 5′ CTG AGC GTC TGT TTG TGT CT 3′
PINCH	Forward 5′ CCG CTG AGA AGA TCG TGA AC 3′
	Reverse 5′ GGG CAA AGA GCA TCT GAA AG 3′
α-Parvin	Forward 5′ CAATTCGACTCCCAGACCAT 3′
	Reverse 5′ TGGTCGAACAAGGTGTCAAA 3′
Mig-2	Forward 5′ AGCTTTATGAGCAGGCCAAA 3′
	Reverse 5′ GAAAGGGCAGCATCAACTTC 3′
Migfilin	Forward 5′ CGAATGCATGGGAAGAAACT 3′
	Reverse 5′ GCAGGTTAGGAAGGGAAACC 3′
β-catenin	Forward 5′ TCATGCGTTCTCCTCAGATG 3′
	Reverse 5′ AATCCACTGGTGAACCAAGC 3′
MMP-13	Forward 5′ TGGCATTGCTGACATCATGA 3′
	Reverse 5′ GCCAGAGGGCCCATCAA 3′
Collagen 10A1	Forward 5′ CAGGCATAAAAGGCCCACTA 3′
	Reverse 5′ GTGGACCAGGAGTACCTTGC 3′
Collagen 2A1	Forward 5′ ATGACAATCTGGCTCCCAACACTGC 3′
	Reverse 5′ GACCGGCCCTATGTCCACACCGAAT 3′
Aggrecan	Forward 5′ TGAGGAGGGCTGGAACAAGTACC 3′
	Reverse 5′ GGAGGTGGTAATTGCAGGGAACA 3′

Fig. 1. MMP-13 is dramatically elevated in OA chondrocytes. (A) Indicative Real Time PCR result for MMP-13 mRNA expression using cDNA isolated from normal and OA chondrocytes. For normal chondrocytes $n = 7$, and for OA chondrocytes $n = 18$. $^*p < 0.05$. (B) Indicative western blot showing MMP-13 protein expression in normal and OA primary chondrocytes. β-Actin is used as loading control.

2.6. Antibodies

Antibodies against ILK [30], Mig-2 [23], and Migfilin [23] were described previously. MMP-13 antibody was purchased from Abcam and β-actin was purchased from Sigma.

2.7. Transfection with siRNAs

Primary osteoarthritic chondrocytes were treated for 24 h with 100 nM siRNA non-specific control or siRNA against Migfilin using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the company's guidelines. Migfilin siRNA sequence was 5′ GAA-GAGGGTGGCATCGTCTGTCTTT 3′, while the sequence 5′ AAACUCUAUCUGCACGCUGAC 3′ was used as Non-Specific Control (NSC).

2.8. Statistical analysis

A paired Student's t-test was used for statistical analyses of the results. p Values <0.05 were considered statistically significant. The Graphpad Prism software was used for the generation of all graphs.

3. Results

3.1. MMP-13 protein and mRNA expression is elevated in OA chondrocyte samples

Primary normal and osteoarthritic chondrocytes isolated from normal and OA articular cartilage, respectively, were first tested for the expression level of MMP-13, a basic catabolic marker used to molecularly identify OA samples. As shown in Fig. 1A and B, OA chondrocytes showed dramatically elevated MMP-13 mRNA (Fig. 1A) and protein (Fig. 1B) expression level compared to normal chondrocytes indicating that they were indeed taken from patients with advanced OA and increased catabolism of ECM. It should be noted however, that although all OA chondrocytes were isolated from patients with advanced OA undergoing joint replacement surgery, they do exhibit differences in MMP-13 protein and mRNA expression level, which is indicative of the variation observed in human samples due to other unidentified factors that may play a role such as genetic predisposition, physical activity, age, and obesity. Thus, for the present study we used chondrocytes which
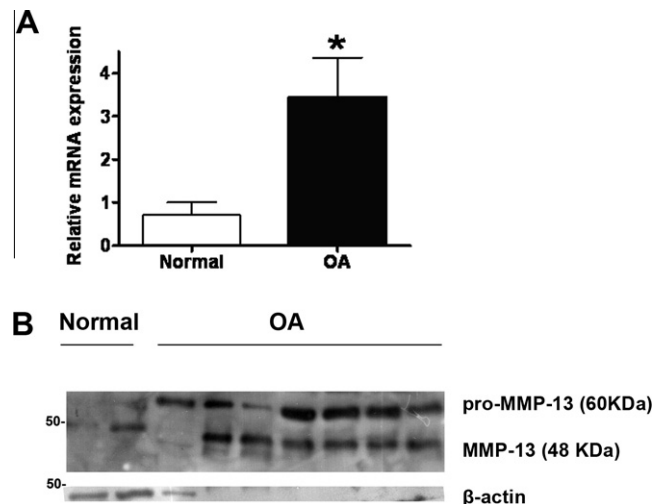
exhibited high mRNA and protein MMP-13 expression at a level that is proportional to one another.

3.2. ILK expression level is elevated in OA chondrocytes but PINCH, α-parvin and Mig-2 are not affected

In order to test whether ILK and its associated proteins are involved in OA pathogenesis, we used normal and OA primary chondrocytes to examine the expression of ILK and its binding partners in the complex ILK-PINCH-parvin. As shown in Fig. 2, ILK protein (Fig. 2A and B) expression was significantly elevated in OA chondrocytes compared to their normal counterparts. In addition, the protein expression level of ILK was also tested in cells taken from a minimally (min) and a maximally (max) affected area of the cartilage tissue as described in Section 2. Fig. 2B shows a clear difference in the expression level between less affected and severely affected cells further suggesting that ILK is also associated with the stage of the disease. Interestingly, however, the mRNA expression of ILK (Fig. 2C) and that of its binding partners PINCH-1 and α-parvin (Fig. 2D), was found to be unaffected in OA, clearly indicating that ILK may exert its action through an alternative pathway in human chondrocytes.

To test whether ILK acts through its interaction with Mig-2 which is a relatively novel ECM-adhesion protein also demonstrated to be associated with it [22], we performed expression analysis for Mig-2 in normal and OA primary human chondrocytes. As shown in Fig. 2E and F, Mig-2 mRNA (Fig. 2F) as well as protein (Fig. 2E) expression level remained unaltered in normal and OA samples.

3.3. Migfilin is upregulated in OA chondrocytes

Although Mig-2 expression was not altered in OA, we moved on to test the expression level of Migfilin which is a Mig-2 and filamin-interacting protein [23]. Migfilin, was an especially promising candidate as recent findings indicated that Migfilin is essential for proper control of bone remodeling [31] while Migfilin knock-out mice exhibited a severe osteopenic phenotype [31]. As shown in Fig. 3A Migfilin protein expression is indeed dramatically elevated in OA samples compared to normal and in fact this difference is also significant between minimally and maximally affected OA
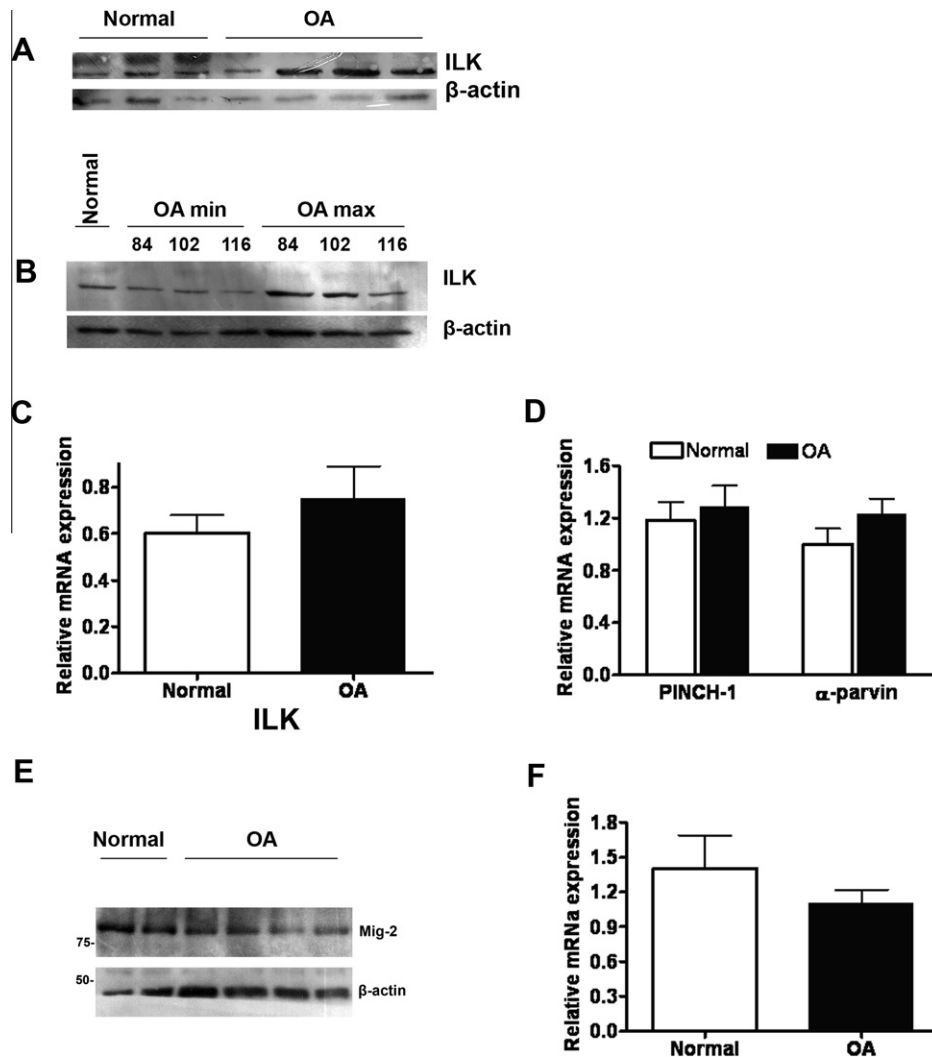
Fig. 2. Expression profile of ILK, PINCH-1, α-parvin and Mig-2 in human chondrocytes. (A) Indicative western blot showing ILK protein expression in normal and OA chondrocytes. (B) Western blot showing ILK protein expression in OA chondrocytes isolated from minimally and maximally affected areas of the same cartilage tissue as described in the materials and methods section. (C) Real Time PCR result for ILK. (D) Real Time PCR result for PINCH-1 and α-parvin. (E) Indicative western blot showing Mig-2 protein expression in normal and OA chondrocytes. (F) Real Time PCR result for Mig-2 mRNA expression in RNA isolated from normal and OA chondrocytes. Diagrams are the result of three independent experiments. For normal chondrocytes $n = 8$, and for OA chondrocytes $n = 18$. $^*p < 0.05$.

samples (Fig. 3B and D), indicating a potentially important role of Migfilin in OA pathogenesis. In addition, Migfilin expression is also elevated at the mRNA level as well (Fig. 3C).

3.4. Migfilin silencing in OA chondrocytes exacerbates osteoarthritic phenotype

Being intrigued by the finding that Migfilin is elevated in OA chondrocytes, we further investigated its role in human OA by gene silencing. More specifically, primary OA chondrocytes were subjected to siRNA trasfection using a non-specific control (NSC) siRNA or Migfilin siRNA. As shown in Fig. 4, Migfilin silencing was successful both at the mRNA (Fig. 4A) and at the protein level (Fig. 4B, compare lanes 1 and 2). Interestingly, Migfilin silencing resulted in significant changes in all the markers normally used to define osteoarthritic state. Thus, Migfilin elimination from osteoarthritic chondrocytes resulted in increased expression of the catabolic marker MMP-13, increased expression of the hypertrophic marker collagen 10A1 and a simultaneous decrease in aggrecan which is abundant in normal chondrocytes. Collagen 2A1 charac-

teristic of normal chondrocytes was also found significantly reduced in OA chondrocytes treated with Migfilin siRNA (Fig. 4A).

3.5. Migfilin silencing leads to β-catenin upregulation

Taking into account recent work by He et al. [32] which showed that Migfilin is inversely correlated with β-catenin, promoting its degradation and combining this piece of information with previous work from our group showing elevated β-catenin expression in OA [33,34], we hypothesized that β-catenin may also be affected by Migfilin silencing. Indeed, as shown in Fig. 4A and B Migfilin depletion led to upregulation of free β-catenin both at the mRNA (Fig. 4A) and the protein (Fig. 4B) level.

4. Discussion

In the present study we evaluated the possible involvement of cell–ECM adhesion-related proteins, such as ILK, PINCH, parvin, Mig-2 and Migfilin in OA pathogenesis. Our findings show that from the proteins examined, only ILK and Migfilin's expression levels were upregulated in OA samples. The observed upregulation
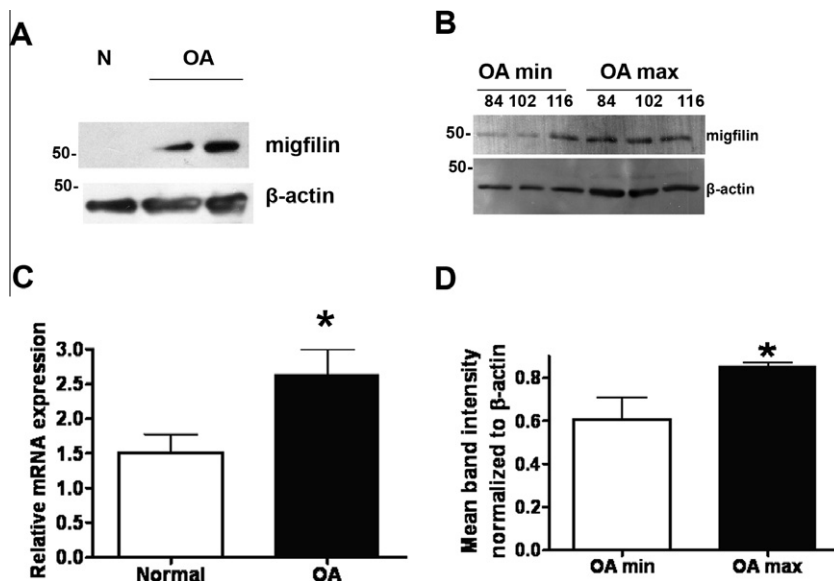
Fig. 3. Migfilin is upregulated in OA chondrocytes. (A) Indicative western blot showing Migfilin protein expression in normal and OA chondrocytes. (B) Western blot showing Migfilin protein expression in OA chondrocytes isolated from minimally and maximally affected areas of the same cartilage tissue as described in Section 2. (C) Real Time PCR result for Migfilin mRNA expression in RNA isolated from normal and OA chondrocytes. Three independent experiments in duplicate were performed using $n = 8$ normal chondrocytes, and $n = 18$ OA chondrocytes ($p = 0.044$). (D) Diagram showing Migfilin protein expression normalized to β-actin using the NIH Scion Image Software. The result shown represents the mean from three different blots ($p = 0.048$). $^*p < 0.05$.
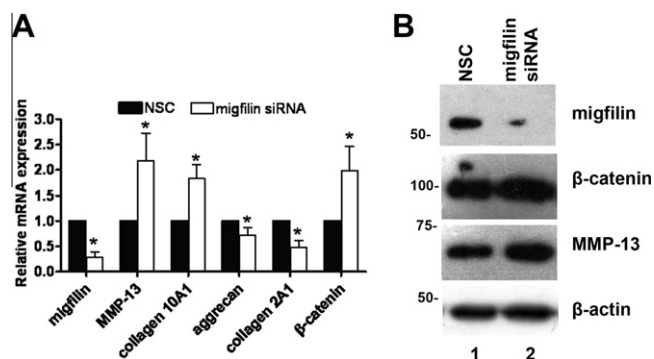

Fig. 4. (A) Real Time PCR results in OA chondrocytes 24 h post-transfection with NSC or Migfilin-specific siRNA. The diagram shows results for the genes: Migfilin ($p = 0.00018$), MMP-13 ($p = 0.027$), collagen 10A1 ($p = 0.001$), aggrecan ($p = 0.03$), collagen 2A1 ($p = 0.0014$) and β-catenin ($p = 0.033$). Diagrams are the result of 4 independent experiments performed in duplicate from 5 different OA samples. Asterisk indicates a statistically significant difference ($p < 0.05$). (B) Indicative Western blot showing Migfilin, β-catenin, and MMP-13 protein expression in OA chondrocytes following Migfilin depletion. β-Actin is used as loading control.

could indicate a possible involvement of both proteins in OA pathogenesis.

Considering the fact that Migfilin knock-out animals exhibit osteopenia [31], and that severe OA is characterized by the formation of new bone in the form of osteophytes [3,5], it makes perfect sense that Migfilin is upregulated in advanced OA. In fact, in the present study we have shown that there is differential protein expression of Migfilin in chondrocytes isolated from severely damaged (OA max) and mildly affected (OA min) areas of the cartilage. As severely damaged cartilage was taken from the main defective areas of maximal load it is considered to depict the most advanced form of the disease. The mildly affected cartilage (OA min) however, was taken from an adjacent macroscopically and histological intact area of the same joint that was clearly involved in the disease process, and thus cannot be completely comparable to normal cartilage nor to severely damaged one. This places the OA min cartilage and chondrocytes in an intermediate state and could be

considered as an earlier stage of the disease. Thus, the findings of the present study indicate that Migfilin's expression is correlated with the disease stage. This is in accordance with previous studies from our group [35], in which several proteins considered to be important for OA pathogenesis (such as leptin) are differentially expressed between maximally and minimally affected chondrocytes.

Since upregulation of Migfilin was more dramatic and consistent than that of ILK among samples tested, and since it occurred in both mRNA and protein level, we decided to further pursue it. Thus, we proceeded with Migfilin silencing in OA chondrocytes and we found that Migfilin elimination rather exacerbates than ameliorates osteoarthritic phenotype. Following Migfilin ablation, OA chondrocytes exhibited even higher expression levels of catabolic and hypertrophic markers (MMP-13, Collagen 10A1 respectively) while exhibiting reduction in ECM molecules (aggrecan and collagen 2A1). This could be explained by a negative feedback-loop mechanism activated by Migfilin in OA chondrocytes.

Furthermore, in the present study we showed that migfillin silencing leads to β-catenin upregulation. This finding is in accordance with reports in cancer cells showing Migfilin to be inversely correlated with β-catenin [31]. Moreover, β-catenin signaling pathway has been recently shown to be involved in OA, as conditional activation of β-catenin in mouse articular chondrocytes leads to premature chondrocytes differentiation and the development of an OA-like phenotype [36]. Recent work from our group demonstrated that β-catenin signaling pathway activated by BMP-2 enhances ECM degradation and promotes hypertrophy as evidenced by collagen 10 expression, suggesting a major role played by β-catenin in OA progression [34]. However, this is the first time that a connection between Migfilin and β-catenin activation is highlighted in OA chondrocytes, adding to the complexity of the underlining mechanisms.

This work highlights the importance of cell–ECM adhesion proteins in OA pathogenesis and provides a rough network of proteins involved. Furthermore, it emphasizes for the first time to our knowledge the role of Migfilin in OA, pointing towards a negative regulatory mechanism of action. However, there are still a few questions that need to be further investigated in relation to the ex-

act molecular mechanism involved. Nevertheless, the present work paves the way for a more thorough investigation of the role of cell–ECM adhesion proteins in OA pathogenesis.

Acknowledgments

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