ADAMTS-5 mRNA levels. This observation is supported by preliminary observations of increased *ADAMTS-5* expression in miR-140 knock out mice (in preparation).

The pathogenesis of osteoarthritis is associated with abnormal activation and differentiation of chondrocytes which overexpress inflammatory mediators and matrix degrading enzymes (3-6). Previous mechanisms examined in these abnormal cellular responses include chondrocyte stimulation by extracellular stimuli such as cytokines, growth factors, mechanical stress and matrix degradation products. Intracellularly these stimuli activate signaling cascades that lead to changes in gene expression (22, 35). This represents a new mechanism by which IL-1 changes gene expression in chondrocytes. MiR-140 represents novel additional control mechanism that is involved in the chondrocyte response to IL-1.

The present study is focused on miR-140 as it is the most cartilage specific miR. We performed searches in three databases ("TargetScan" http://www.targetscan.org/vert_50/ , " PicTar" http://pictar.mdc-berlin.de/, "miRanda" http://microrna.sanger.ac.uk/) and this yielded 223-975 potential miR-140 targets. Only 9 potential targets were identified in all three databases, and notably, this did not include ADAMTS-5. There remains uncertainty in regards to the rules for in silico miR target identification (36). At present the most conclusive target validation is the demonstration of changes in protein expression, cell function or phenotype in knock out or transgenic mice. Future studies are needed to determine the consequences of changes in the compete set of miR-140 targets for cartilage development and homeostasis. Currently ongoing studies with miR-140 knock out mice and miR-140 transgenic mice will provide information in this regard.

In conclusion, our study suggests that miR-140 is a chondrocyte differentiation-related miR. It may be novel regulator of cartilage homeostasis and changes in its expression and

function play an important role in diseases affecting articular cartilage. Further studies on miR-140 have the potential to reveal important new regulatory pathways that control cartilage development and homeostasis and open a new insight on disease mechanisms and therapeutic interventions for OA.

ACKNOWLEDGEMENTS

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miRNA	Ratio (Chondrocyte/MSC)	*Chondrocyte	*hMSC
miR-140	2.12	540.89	254.377
miR-197	1.88	5395.03	2869.38
miR-148	1.78	644.35	361.58
miR-328	1.70	2768.41	1632.95
miR-27b	1.63	6092.21	3746.00
miR-16	1.59	4398.78	2764.09
miR-222	1.55	6349.65	4087.27
miR-15b	1.55	668.29	430.56
miR-505	1.54	1967.68	1273.77
miR-23b	1.52	8114.26	5334.24

Table. 1 miR expression in human articular chondrocytes and MSCs. RNA was isolated from articular chondrocytes and MSCs for microarray analysis of miRs. Differentially expressed (at least 1.5 fold difference) miRs are shown as ratio chondrocytes/MSCs. *raw signal intensity values.

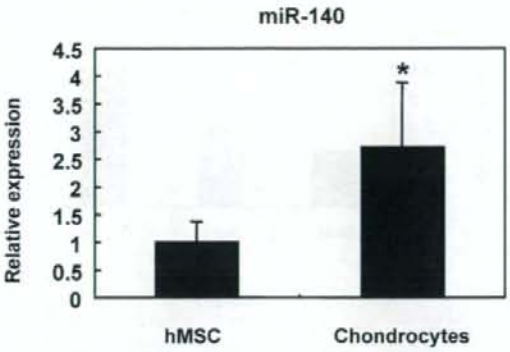

Figure 1. The expression of miR-140 in articular chondrocytes and MSCs. Array data for miR-140 were validated by qPCR on MSCs (n=3, different preparations from 2 different donors) and articular chondrocytes (n=8, different preparations from 8 different donors). miR-140 expression was significantly higher in chondrocytes compared to MSCs (*P=0.015). Values are mean ±SEM expressed as fold difference relative to expression level in MSCs.

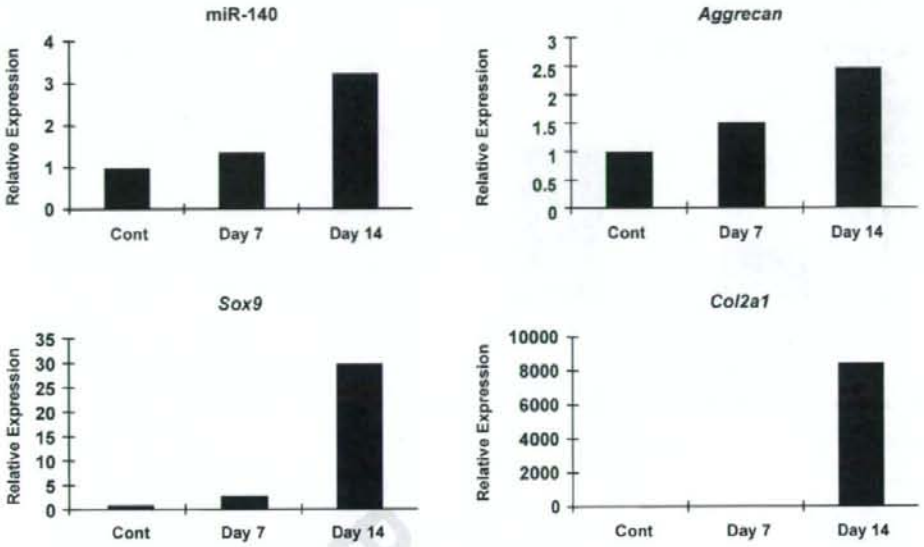

Figure 2. Changes in miR-140 expression during chondrogenesis. RNA was isolated from undifferentiated MSCs (cont) and from MSCs pellets cultures in chondrogenesis medium after 7 and 14 days. miR-140, *Sox9* and *Col2a1*expression were analyzed by qPCR. The expression of miR-140 increased during chondrogenesis along with increased *Sox9* and *Col2a1*. Results are shown as relative expression where expression in undifferentiated MSCs (cont) is defined as 1.

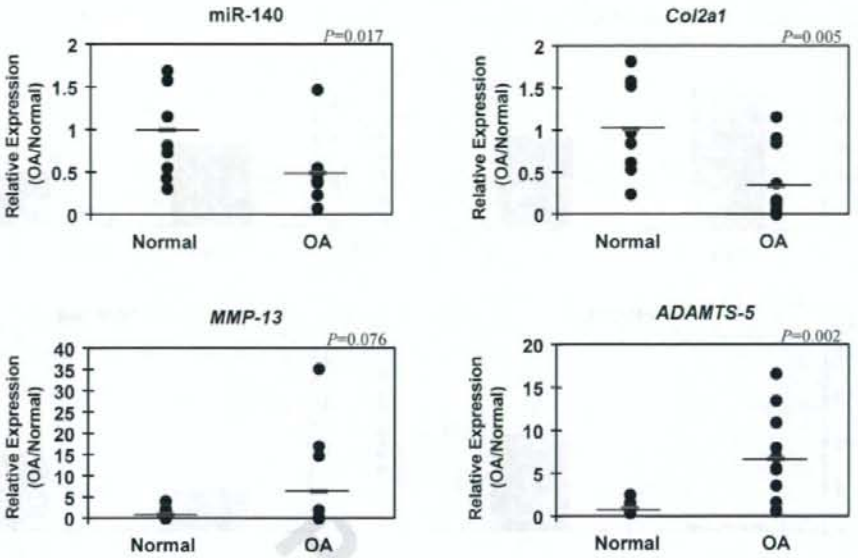

Figure 3. miR-140 expression in normal and OA articular cartilage. Full thickness cartilage was collected from normal (n=8) and OA (n=11) knee joints for RNA isolation. miR-140, *Col2a1*, *ADAMTS-5*, and *Sox9* expression were determined by qPCR. miR140 (P=0.017) and *Col2a1* (P=0.005) expression was significantly decreased and *ADAMTS-5* (P=0.002) was significantly increased in OA cartilage compared with normal cartilage. Values are mean ±SEM expressed as fold difference relative to normal cartilage expression level.

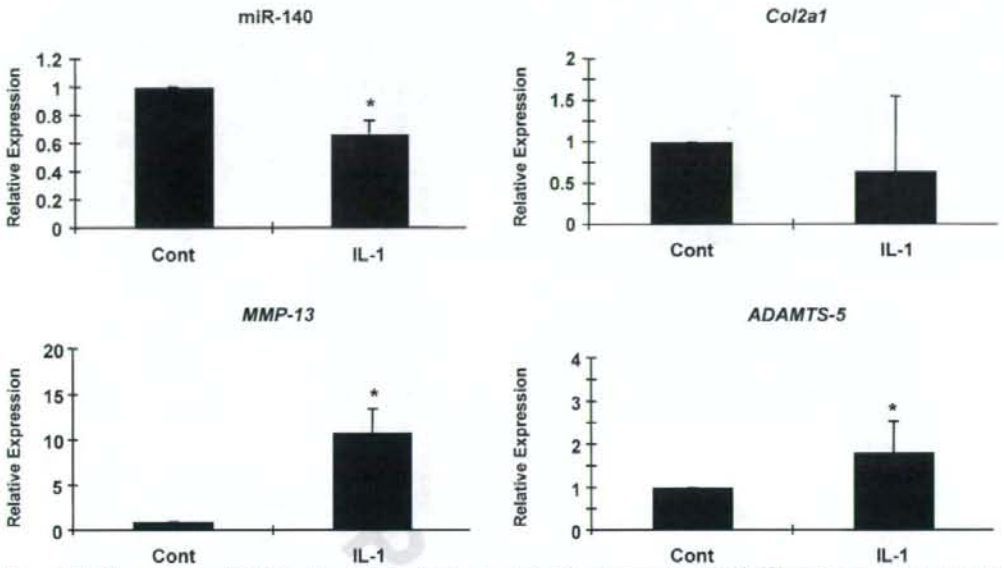

Figure 4. IL-1ß suppresses miR-140 in vitro. Articular chondrocytes (n=8 different preparations from 8 different donors) were treated with IL-1ß (5 ng/ml) for 5 hours. miR-140, *Col2a1*, *MMP-13* and *ADAMTS-5* were analyzed by qPCR. IL-1ß stimulation significantly decreased miR-140 expression and increased *MMP-13* and *ADAMTS-5* expression. *Col2a1* expression did not significantly change. Values are mean ±SEM expressed as fold difference relative to control expression level. *Significant difference ($p<0.05$).

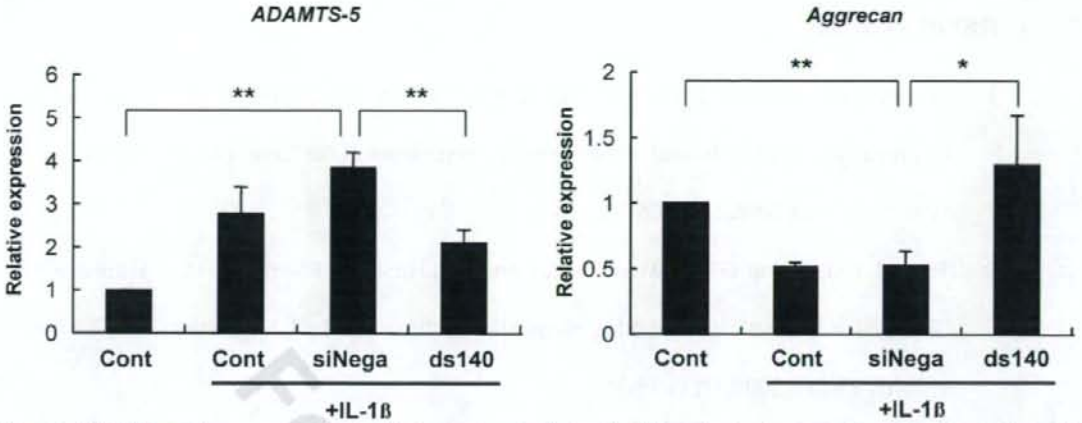

Figure 5. *ADAMTS-5* and *Aggrecan* expression in articular chondrocytes by ds-miR-140. Articular chondrocytes (n=3) were transfected with ds-miR-140. *ADAMTS-5* and *Aggrecan* expression were analyzed by qPCR. (A) The ds-miR-140 significantly reduced *ADAMTS-5* expression with and without IL-1ß (5 ng/ml) stimulation for 5 hours. (B) The decreased *Aggrecan* expression with IL-1ß stimulation was significantly increased by ds-miR-140. Values are mean ±SEM expressed as fold difference relative to siNegative control expression level. Significant difference (**=$p<0.01$, *= $p<0.05$).

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Expression of microRNA-146 in osteoarthritis cartilage

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Abstract

Objective. A role of microRNAs, which are ~22- nucleotide non coding RNAs, has recently been recognized in human diseases. The objective of this study was to identify the expression pattern of microRNA-146 (miR-146) in cartilage from patients with osteoarthritis (OA).

Methods. The expression of miR-146 in cartilage from 15 patients with OA was analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and by *in situ* hybridization. Induction of the expression of miR-146 by cultures of normal human articular chondrocytes following stimulation with interleukin-1β (IL-1β) was examined by quantitative RT-PCR.

Results. All cartilage samples were divided into three groups according to a modified Mankin scale; grade I: 0 - 5, grade II: 6 - 10, grade III: 11 - 14. In OA cartilage samples of grade I, the expression of miR-146a and Col2a1 was significantly higher than that of other groups ($p<0.05$). In OA cartilage of grades II and III, the expression of miR-146a and Col2a1 decreased while the expression of MMP13 was elevated in grade II. These data show that miR-146a is expressed intensely in cartilage with a low Mankin grade, and that miR-146a expression decreases in accordance with level of MMP13 expression. Section *in situ* hybridization of pri-miR-146a revealed that pri-miR-146a is expressed in chondrocytes in all layers, especially in the superficial layer where it is intensely expressed. The expression of miR-146 was markedly elevated by IL-1β stimulation in human chondrocytes in vitro.

Conclusion. This study shows that miR-146 is intensely expressed in low grade OA cartilage, and that its expression is induced by stimulation of IL-1β. MiR-146 might play a role in OA cartilage pathogenesis.

Introduction

Osteoarthritis (OA) is a highly prevalent disease, which is characterized by progressive degeneration of articular cartilage [1-4]. Although little is known about OA pathogenesis, an imbalance between anabolic and catabolic factors which maintains the homeostasis of cartilage is thought to lead to cartilage degradation. While there is a delicate balance between anabolism and catabolism, strictly regulating matrix turnover in normal cartilage, catabolism becomes dominant over anabolism in OA cartilage, leading to the degradation of cartilage. Several reports have demonstrated an interaction between anabolic factors such as TGF-β and catabolic factors such as matrix metalloproteinase and aggrecanase in chondrocytes, however, the molecular mechanisms involved in OA remain unclear [5].

MicroRNA (miRNA)s are a family of ~22-nucleotide non coding RNAs identified in organisms ranging from nematodes to humans [6-8]. Many miRNAs are evolutionarily conserved across phyla, regulating gene expression by posttranscriptional gene repression. The miRNAs regulate gene expression by binding the 3'-untranslated region of their target mRNAs leading to translational repression or mRNA degradation. Several microRNAs exhibit a tissue-specific or developmental stage–specific expression pattern and have been reported to be associated with human diseases such as cancer, leukemia, and viral infection [9-11].

Taganov *et al.* reported that miRNA-146a/b (miR-146a/b) is induced in response to lipopolysaccharide (LPS) and proinflammatory mediators in THP-1 cells and that its induction is regulated by nuclear factor –kappa B (NF-κB) [12]. Nakasa *et al.* reported that miR-146 is expressed more intensely in synovial tissues of rheumatoid arthritis compared to that of OA and normal individuals, and its expression in rheumatoid arthritis synovial fibroblasts was induced by stimulation with inflammatory cytokines such as TNFα and IL-1β[13]. Inflammatory cytokines also play an important role as catabolic factors in OA cartilage [14]. Therefore, there is the possibility that miR-146a might be expressed in OA cartilage and thus participate in the anabolic and catabolic balance. The aim of this study is to identify the expression of miR-146a in OA cartilage from OA patients, and its induction by IL-1β in human chondrocytes.

Patients and methods

Patients

Articular cartilage samples were obtained from 15 OA patients (64.3 ± 15.7 years of age, mean ± SD) undergoing operations. OA was diagnosed according to the American Rheumatism Association Criteria for OA. Nine patients with affected hips underwent total arthroplasty and six patients with affected knees underwent total knee arthroplasty with the exception of patient 1 who had secondary OA in the patellofemoral joint following trauma injury to the articular cartilage, and underwent arthroscopic debridement. Their clinical characteristics are shown in Table 1.

This clinical research was conducted in compliance with the Declaration of Helsinki. Written permission was obtained from all patients who participated in this study.

Tissue samples

Cartilage specimens, including all cartilage layers and subchondral bone, were obtained from the load bearing sites of the femoral condyles or femoral head except for patient 1. Cartilage from patient 1 was obtained from the patella. Total cartilage loss sites were avoided. Articular cartilage was dissected into 2 parts; one was used for the isolation of RNA, the other for histology. For polymerase chain reaction (PCR) analysis, total RNA was isolated from cartilage that had been homogenized on ice with Trizol reagent (Invitrogen). For histologic analysis, cartilage samples were fixed in 4% paraformaldehyde (PFA), decalcified in 0.24 M EDTA (Sigma-Aldrich, Japan) at 4 ℃ until the bones were pliable, then embedded in paraffin.

Synthesis of complementary DNA (cDNA)

Total RNA yields were calculated and quality was determined using absorption spectrochemical analysis. One

microgram of total RNA was reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The genomic DNA elimination reaction was carried out using 2μl of gDNA wipeout buffer, 1μg (1μl) template RNA and 11μl RNase-free water at 42°C for 2 min. Reverse transcription was performed in 1μl quantiscript reverse transcriptase, 4μl quantiscript RT buffer, 1μl RT primer mix and 14μl template RNA (the entire genomic DNA elimination reaction) at 42°C for 15 min and 95°C for 3 min and then the cDNA product was maintained at 4°C.

Quantitative (real-time) PCR

Quantitative reverse transcription–PCR (RT-PCR) assays were performed using a TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA) for the mature miR-146a and **a** Taqman Gene Expression Assay for hCol2a1, hMMP13, and hGAPDH (Applied Biosystems). RT reactions **for** mature miR-146a contained a sample of total RNA, 50 nM stem-loop RT primer, 10× RT buffer, 100 mM each dNTPs, 50 units/μl of MultiScribe reverse transcriptase, and 20 units/μl of RNase inhibitor. Reaction mixtures (15μl) were incubated in a thermal cycler (BioRad, Hercules, CA) for 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C and then maintained at 4°C.

Real-time PCR was performed using a Mini Opticon Real-time PCR System (BioRad, Hercules, CA) in a 10 μl PCR mixture containing 2μl of RT product, 5μl of 2× TaqMan Universal PCR Master Mix, 0.2 μM TaqMan probe, 15 μM forward primer, and 0.7 μM reverse primer.

All reactions were incubated in a 48-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute; all were performed in triplicate. The U18 and GAPDH genes were used as controls to normalize differences in total RNA levels between samples. A threshold cycle (C_t) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the $\Delta\Delta C_t$ values and were expressed as $2^{-\Delta\Delta C_t}$. The value of each control sample was set at 1 and was used to calculate the fold change in target genes for the

induction of miR-146a by IL-1β.

Histological analyses

Each paraffin embedded cartilage sample was sectioned at 5 μm and every tenth section was stained with Safranin O-fast green staining. Two independent assessors (TN and KY) graded each sample using a modified Mankin scale [15, 16]. For *in situ* hybridization, primary miR-146a fragments were derived from PCR products, cloned using the Qiagen PCR cloning kit into the pDrive vector (Qiagen, Chatsworth, CA), and sequenced. Primer sequences for primary miR-146a were 5'-TAT-TGG-GCA-AAC-AATCAG-CA-3' (forward) and 5'-GCC-TGA-GAC-TCT-GCCTTC-TG-3' (reverse). Digoxigenin (DIG)–labeled riboprobes were transcribed with a DIG RNA labeling kit and T7 polymerase (Roche, Mannheim, Germany). After deparaffinization, each section was fixed in 4 % paraformaldehyde for 10 minutes at room temperature, washed 3 times in phosphate buffered saline (PBS) for 3 minutes, and subsequently treated with 600 μg of proteinase K for 10 minutes at room temperature. After treatment in 0.2 % glycine-PBS for 10 minutes, sections were refixed in 4 % paraformaldehyde for 10 minutes, washed 3 times in PBS for 3 minutes each, and acetylated with 0.25 % acetic anhydride in 0.1 M triethanolamine hydrochloride for 10 minutes. After washing in PBS for 30 minutes, sections were prehybridized for 1 hour at 65°C with prehybridization buffer (50 % formamide and 5× saline–sodium citrate [SSC]). Hybridization with DIG-labeled riboprobes was performed overnight at 65°C in hybridization buffer (50 % formamide, 5×SSC, 5× Denhardt's solution, and 250 μg/ml of baker's yeast transfer RNA). After hybridization, sections were washed in 5× SSC for 30 minutes at 65°C, 0.2× SSC for 2 hours at 65°C, and 0.2× SSC for 5 minutes at room temperature. Blocking was performed overnight at 4°C with 4 % horse serum and alkaline phosphatase–conjugated Fab anti-DIG antibody (Roche) in 1 % sheep serum. Staining was performed using BCIP and nitroblue tetrazolium (NBT; Roche).

Cell culture

Articular cartilage was harvested from femoral condyles and tibial plateaus of human tissue donors. All articular cartilage samples were graded according to a modified Mankin scale, and we used only chondrocytes from normal articular cartilage. Human chondrocytes were isolated and cultured as described previously [15]. The cartilage tissue was incubated with trypsin at 37°C for 10 minutes. After the trypsin solution was removed, the tissue slices were treated for 12 to 16 hours with type IV clostridial collagenase in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS). After initial isolation, the cells were kept in high-density cultures in DMEM (high glucose) supplemented with 10% CS, L-glutamine, and antibiotics. After the cells had grown to confluence, they were split once (passage 1) and grown to confluence again for use in the experiments.

Induction of miR-146 by stimulation with IL-1β

Cells were seeded at 1.0×10^5/well into a 6-well plate containing 2 ml of DMEM plus 5% FCS and 1% penicillin/streptomycin. After cells became adherent, they were treated with recombinant human IL-1β (5 ng/ml) (PeproTech, Rocky Hill, NJ) and then incubated for 24 hours under an atmosphere of 5 % CO_2. Cells were washed twice with cold PBS, and then total RNA was isolated with Trizol reagent. Real-time PCR was performed in triplicate with the TaqMan microRNA assay kit for mature miR-146a or the Taqman Gene Expression Assay for MMP13.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis was used to compare gene expression between the three groups. The Mann-Whitney U test was used to compare the gene expression between two groups. P values less than 0.05 were considered to be statistically significant. All statistical analyses were performed on a personal computer using the Stat View version 5.0 statistical package (Abacus Concepts, Berkeley, CA).

Results

Expression of mature miR-146a, Col 2A1 and MMP-13 in OA cartilage

The expression of mature miR-146a and of mRNA for Col2a1 and MMP13 were screened by real time PCR in all samples to investigate miR-146a expression and its relationship to the extent of degradation of cartilage in articular cartilage (Figure 1A). All cartilage samples were divided into three groups according to a modified Mankin scale; grade I: 0 - 5, grade II: 6 - 10, grade III: 11 - 14. In OA cartilage samples of grade I, the expression of miR-146a and col2a1 was significantly higher than that of other groups ($p<0.05$) (Figure 1B). In addition, the expression of MMP13 was significantly lower than that of other groups ($p<0.05$). In OA cartilage samples of grades II and III, the expression of miR-146a and col2a1 decreased while the expression of MMP13 increased in grade II. In OA cartilage **of** grade III, MMP13 expression decreased, and there was a significant difference between grades I and II ($p<0.05$). These data indicate that miR-146a is expressed intensely in cartilage with a low grade of the Mankin scale, and miR-146a expression decreases with increasing MMP13 expression.

Distribution of miR-146a in osteoarthritis cartilage

To examine the distribution of cells expressing miR-146a in OA cartilage, we performed *in situ* hybridization (ISH) of primary-miR-146a. ISH revealed that miR-146a is expressed in superficial, middle, and deep layers (Figure 2A-E). The chondrocytes expressing miR-146a were frequently located in the superficial and middle zones, where proteoglycans were depleted from the matrix (Figure 2F-H). In cartilage with the low expression level of miR-146a together with a high level of expression of MMP13, the cells expressing miR-146a were sparsely distributed in comparison with those in cartilage with a high expression level of miR-146a. In clustered chondrocytes, miR-146a also expressed (Figure 2C). In cartilage with a high grade of the Mankin scale, there were few miR-146a expressing cells (Figure2I, J).