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Identification of Genes Regulated by IL-1β Using Integrative microRNA and mRNA Genomic Analysis in Human Articular Chondrocytes

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Objective. **The physiological and pathogenetic role of microRNAs (miRNAs) in the maintenance of joint homeostasis and in the development of arthritis is recently being elucidated. In this study, we attempted to identify differentially expressed miRNAs in human osteoarthritis (OA) chondrocytes in response to interleukin (IL)-1β. In addition, simultaneous profiling of miRNA and mRNA expression was performed to get an integrated analysis of miRNA and mRNA expression.**

Methods. **Monolayer cultured chondrocytes obtained from knee cartilages of OA patients were stimulated with IL-1β for 4 hours and RNA was isolated. One microgram of total RNA was polyadenylated and converted to cDNA and miRNA microarray was performed. Seven hundred thirty five oligos were used, corresponding to 470 well-annotated human miRNA sequences and 265 potential miRNAs that were identified recently. mRNA microarray was performed simultaneously using the RNA samples that were used for miRNA array. Both sequence and expression information was used to identify regulatory relationship between miRNA and mRNA pairs.**

Results. **Expression profiling of miRNA extracted from IL-1β treated chondrocytes identified 25 miRNA which showed differential expression. We also identified 7190 mRNAs differentially regulated by IL-1β treatment. Among the 25 miRNAs differentially regulated, 13 miRNAs had targets searched by MiRANDA scheme. By combining target search and miRNA-mRNA pairing, we could identify 1043 miRNA-mRNA target pairs. MiR-200a was found to be expressed in human OA and normal cartilages, with downregulation in OA lesion cartilages.**

Conclusion. **It is suggested that miRNA may play a role in the regulation of cartilage degradation in OA.**

Key Words. **Chondrocytes, Interleukin-1, microRNA, miR-200a, mRNA, Osteoarthritis**

Introduction

Osteoarthritis (OA) is a degenerative disease of articular cartilage and causes significant morbidity in humans. It is characterized by loss of articular cartilage matrix, mainly collagen and proteoglycans, leading to tissue destruction and cell death, eventually resulting in loss of joint function. Although OA is frequently regarded as a noninflammatory form of arthritis, considerable data implicate a role of proinflammatory cytokines derived from both the synovium and the chondrocytes in cartilage destruction. The proinflammatory cytokine interleukin (IL)-1β and its downstream mediators lead to the upregulation of matrix metalloproteinase (MMP), and a decrease in the synthesis of cartilage extracellular matrix (ECM) (1). IL-1β also induces a cascade of intracellular intermediates that are postulated as important players in the inflammatory pathway of OA, such as inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and phospholipase A_2 (2). A variety of therapeutic strategies for OA have thus been developed to antagonize the activity of IL-1β and its downstream targets. A detailed analysis of the extent of regulation of chondrocyte function by IL-1β would be indispensable for such strategies to be effective and safe.

MicroRNA (miRNA)s are short endogenous oligonucleotides (~22 bp) with a profound role in the regulation of post-transcriptional gene expression. miRNAs regulate their targets both by translational inhibition and acceleration of mRNA degradation (3). Target genes are determined by sequence complementarity between the 3'-untranslated region (UTR)

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and the mature miRNA, particularly in a 6bp 'seed' region (4). Currently, 1,100 human miRNAs are registered in the miRBase database (release 16, September 2010) and each miRNA is expected to target around 200 transcripts (5). Predictive algorithms suggest that up to a third of all human genes contain putative miRNA recognition elements (6). The finding that miRNAs regulate multiple genes and the demonstration of differential expression of miRNA in animal cells and tissues has led to speculation that their control of gene expression is conceptually similar to the action of transcription factors although they act at the translational level (7).

Evidences have been accumulated recently that miRNAs play critical roles in diverse biological processes including development and cancer. Since miRNAs can affect mRNA levels, they might leave a signature on the mRNA expression profile in a specific tissue or biological condition. Therefore, it is suggested that simultaneous profiling of miRNA and mRNA expression may be a plausible way to predict functional miRNA targets. Previously, it was reported that paired expression profiling of miRNA and mRNA identify a network of 1597 high-confidence target predictions for 104 human miRNAs across 88 tissues and cell types with high precision (8).

In this study, we attempted to identify differentially expressed miRNAs in human OA chondrocytes in response to IL-1β. In addition, simultaneous profiling of miRNA and mRNA expression was performed for an integrated analysis of miRNA and mRNA expression.

Materials and Methods

Reagents

Dulbecco's modified Eagle medium (DMEM), fetal calf serum, and Fungizone were obtained from Gibco (Grand Island, NY). Human recombinant IL-1β was obtained from R & D Systems (Minneapolis, MN). TRIzol was obtained from Invitrogen Life Technologies (Carlsbad, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Chondrocyte monolayer culture

Cartilage samples were obtained from the femoral condyle and tibial plateau of the knee from OA patients at the time of joint replacement surgery. The collection and use of human samples were reviewed and approved by the Institutional Review Board of Hallym University Sacred Heart Hospital (Anyang, Korea). Full-thickness cartilage slices were obtained from above the subchondral bone from a relatively lesion-free area. Chondrocytes were cultured in monolayer as was described previously (9). Briefly, slices were minced and incubated sequentially with pronase and collagenase and released cells were seeded at a density of 5×10^6/plate in 10 cm culture plates in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% Fungizone and penicillin/streptomycin (150 units/mL and 50 mg/mL each). After about 7 days, confluent chondrocytes were split once, seeded at high density and these first passage chondrocytes were used within 2 days in subsequent experiments. Chondrocytes were incubated with DMEM containing 0.5% fetal calf serum for 16 hours prior to treatment with IL-1β (1ng/mL).

RNA preparation

RNA was isolated with TRIzol from cultured chondrocytes 4 hours after treatment with IL-1β, and purified using mirVANA kit according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA). For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis and optical density (OD) 260/280 ratio.

microRNA and mRNA microarray

One microgram of total RNA was polyadenylated and converted to cDNA using poly-A polymerase, polyadenylation reaction reagent, and cDNA synthesis reagent (Illumina, San Diego, CA). One miRNA-specific oligo containing a universal PCR priming site, an address sequence that complements a corresponding capture sequence on the array, and a microRNA-specific sequence was used to assay each microRNA. Seven hundred thirty five oligos were used, corresponding to 470 well-annotated human microRNA sequences and 265 potential microRNAs that were identified recently. After labeling the DNA with fluorolink streptavidin-Cy3 (Molecular Probes, Carlsbad, CA), hybridization was conducted under a temperature gradient program from 60°C to 45°C over 14 hours. After hybridization, the arrays were rinsed and then imaged at a resolution of 0.8 microns using a BeadArray Reader (Illumina). For mRNA microarray, the same RNA samples used in miRNA microarray were amplified and purified using the RNA amplification kit (Ambion, Austin, TX) to yield biotinylated cRNA according to the manufacturer's instructions. Seven hundred fifty nanogram of labeled cRNA samples were hybridized to each human HT-12 expression bead array for 16～18 h at 58°C, according to the manufacturer's instructions (Illumina). Detection of array signal was carried out using Cy3 following the bead array manual.

Microarray data analysis

For both mRNA and miRNA expression arrays, the quality of hybridization and overall chip performance were monitored

by visual inspection of both internal quality control checks and the raw scanned data. To identify differentially expressed mRNA and miRNA, we used Student t-test. To adjust multiple testing problems, we estimated the proportion of false positives by using the false discovery rates which was controlled by Benjamini and Hochberg algorithm (10). All of statistical analyses were performed using R statistical language.

Sequence & expression based miRNA target prediction

We used both sequence and expression information to identify regulatory relationship between miRNA and mRNA pairs. We downloaded miRNA-mRNA target pair data estimated by miRanda algorithm (11), based on local sequence alignment. Among differentially expressed miRNAs and mRNAs that were identified by statistical analysis of microarray data, we searched for the miRNA-mRNA target pairs predicted by miRanda algorithm.

Reverse transcription polymerase chain reaction (RT-PCR)

To validate the miRNA microarray data, quantitative real-time RT-PCR analysis for selected miRNA was performed. Total RNA was isolated from IL-1β treated monolayer chondrocytes obtained from donors different from those that were used in miRNA microarray. Expression of miR-200a was assessed using a TaqMan MicroRNA Assay protocol (Applied Biosystems). cDNA was synthesized from 2 μg of total RNA using miRNA specific primers and the TaqMan MicroRNA Reverse Transcription Kit (both from Applied Biosystems). Real-time RT-PCR was performed using an Applied Biosystems 7900HT Sequence Detection System in a 20-μL PCR mixture containing 1.33 μL of RT product, 10 μL TaqMan Universal PCR Master Mix, 1 μL Taqman microRNA assay reagent containing forward and reverse primers and 7.67 μL nuclease free water. All reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 1 second; all reactions were performed in triplicate. The RNA, U6 small nuclear 2 (RNU6-2) gene was used as a control to normalize differences in total RNA levels in each sample. 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 $2^{-\Delta\Delta Ct}$. Data are given as fold changes of average signals in control and IL-1β treated chondrocytes. mRNA microarray data were validated by semi-quantitative RT-PCR analysis for selected genes. Total RNA was isolated from IL-1β treated monolayer chondrocytes obtained from donors different from those that were used in mRNA microarray. To assess the expression of TNS1 and Tbx15, cDNA was synthesized from 2 μg of total RNA using MMLV reverse transcriptase (Promega, WI, USA). One μL of the RT product was used as a template for PCR amplification of TNS1, Tbx15, and GAPDH using the following cycles: TNS1 and Tbx15, 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 minute for over 26～28 cycles; GAPDH, 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 minutes for over 20 cycles. Primer pairs were as follows: TNS1, sense 5'-TAAACAGCTCGGCTTGTCCT-3' and antisense 5'-GGAGCAACTTGCCTTTCTTG-3'; Tbx15, sense 5'-TAACCAACAGGCAACCAACA-3' and antisense 5'-CTCT TCAAAGGCCACTCTGG-3'; GAPD H, sense 5'-GAAGGTGA AGGTCGGAGTCA-3' and antisense 5'-GAAGATGGTGATGG GATTTC-3.' PCR products were separated by electrophoresis on a 1.5% agarose gel and were visualized by ethidium bromide staining.

In situ hybridization

Cartilage tissues obtained from OA patients at the time of joint replacement surgery were cut to the subchondral bone from both lesion and non-lesion areas (defined as areas with preserved cartilage surface and thickness), immediately fixed with 4% paraformaldehyde and embedded in paraffin. For non-OA cartilage, macro and microscopically normal cartilage samples obtained from the femoral head of patients with femoral neck fracture were used. After dewaxing and dehydration, sections were washed in phosphate buffered saline (PBS) and deproteinated with proteinase K (10 μg/mL at 37°C). Sections were washed once in 0.2% glycine in PBS and twice in PBS. Prehybridization was performed in a humidified chamber (50% formamide, 5X SSC) using hybridization buffer (50% formamide, 5X SSC, 0.1% Tween, 9.2 mM citric acid adjusted to pH 6, 50 μg/mL heparin, and 500 μg/mL yeast RNA) for 2 hours. Slides were then hybridized overnight with 20 nM of FITC-labeled probes for miR-200a (CACA AACCATTATGTGCTGCTA) or scrambled miRNA (TTCAC AATGCGTTATCGGATGT) in a humidified chamber at 65°C. Sections were rinsed in 5x SSC for 30 minutes at 65°C, 0.2x SSC for 2 hours at 65°C, and 0.2x SSC for 5 minutes at room temperature.

Data analysis

Data are expressed as means±SDs. Differences between treatment groups were tested by using the Mann-Whitney U test (GraphPad Prism, version 3, GraphPad Software, San Diego, CA). Significance was established at the 95% confidence level ($p < 0.05$).

Table 1. MiRNAs with differential expression between IL-1β treated and control chondrocytes from OA cartilages (n=6)

miRNA	Chromosomal location	Fold change
tHS_15.1	Unknown	2.15
HS_176	11	2.01
HS_221	15	1.63
hsa-miR-202	10	1.52
hsa-miR-335	7	1.48
hsa-miR-563	3	1.46
hsa-miR-362	×	1.39
hsa-miR-452	×	1.38
HS_149	9	1.36
hsa-miR-603	10	1.35
HS_175	11	1.29
hsa-miR-425	3	1.24
HS_219	15	1.16
hsa-miR-507	×	1.16
hsa-miR-766	×	1.15
HS_145.1	9	1.08
hsa-let-7e	19	1.04
hsa-miR-31	9	-1.06
hsa-miR-454-3p	17	-1.11
hsa-miR-181b	9	-1.25
hsa-miR-565	3	-1.28
hsa-miR-200a	1	-1.42
hsa-miR-449	5	-1.42
HS_141	10	-1.46
hsa-miR-594	7	-1.53

Positive fold change values indicate elevated expression and negative fold change values indicate decreased expression in IL-1β treated chondrocytes compared to control.

Results

Expression profiling of 735 human miRNA extracted from IL-1β treated chondrocytes (n=6) identified 25 miRNA which showed differential expression between control and treated cells (Table 1). Overall, the difference in expression was modest, and only 2 miRNAs showed more than 2 fold difference in expression level between control and treated samples (HS_176, tHS_15.1). We also identified 7190 mRNAs differentially regulated by IL-1β treatment. Large numbers of mRNA encoding proinflammatory and catabolic mediators previously reported to be regulated by IL-1β were identified by the mRNA microarray, including MMP-13, inducible nitric oxide synthase, IL-6, tumor necrosis factor (TNF)-alpha and various chemokines (data not shown). Among the 25 miRNAs differentially regulated, 14 miRNAs had targets searched by MiRANDA scheme. By combining target search and miRNA-mRNA pairing, we could identify 1043 miRNA-mRNA target pairs. Table 2 and 3 shows selected pairs of miRNA-mRNA relevant in the cartilage biology and arthritis.

We selected one downregulated miRNA, miR-200a and examined their expression in human articular chondrocytes and cartilages. In line with the miRNA microarray result, monolayer cultured OA chondrocytes treated with IL-1β showed downregulation of miR-200a, when miRNA level was examined by real-time RT-PCR (Fig. 1). The expression levels of 2 selected target genes for miR-200a, T-box 15 (tbox-15), a transcription factor regulating proliferation of chondrocytes (52), and tensin

Table 2. MiRNAs upregulated by IL-1β and their target mRNAs identified by integrative analysis

MicroRNA (upregulated)	mRNA (downregulated)	Comments
Let-7e	GSPT1 (G1 to S phase transition 1)	Controls G1 to the S phase transition; downregulated in chondrocyte redifferentiation in 3D pellet culture (12)
	SOCS1 (suppressors of cytokine signaling 1)	Induced by FGF; up-regulated in the synovial membranes from patients with RA (13)
	SNX19 (sorting nexin 19)	Expressed in the limb cartilage of mouse embryos and in the degraded cartilage of adult mouse knee joints during osteoarthritis progression; a potent chondrogenic stimulator (14)
	CLP1 (cleavage and polyadenylation factor I subunit homolog)	Stimulates cell proliferation in rabbit articular chondrocytes (15)
miR-335	CASP7 (caspase7)	Elicited in human articular chondrocytes by sodium nitroprusside (16)
	Hoxd13 (homeobox D13)	Development of the forelimb and hindlimb autopod in mice (17)
	TLR1 (Toll-like receptor 1)	Pattern recognition receptor involved in innate immunity
	TIAM1 (T-cell lymphoma invasion and metastasis 1)	Down-regulated in high pressure culture and upregulated in cyclic stretching culture in chondrosarcoma line (18); linked with hyaluronan metabolism (19)
miR-425	TNC (Tenascin)	Oligomeric glycoprotein of the extracellular matrix; upregulated in tissues at developmental stages, during wound healing, and during tumorigenesis; upregulated in neonatal cartilage (20)
	IL4R (IL4 receptor)	Involved in the anabolic response of chondrocytes from normal articular cartilage; chondroprotective in cartilage biology and play a central role in the maintenance of the extracellular matrix (21)

Table 2. Continued

MicroRNA (upregulated)	mRNA (downregulated)	Comments
	VIM (Vimentin)	A member of the intermediate filament family, which make up the cytoskeleton; maintains cell shape, integrity of the cytoplasm, and stabilizes cytoskeletal interactions; decreased in chondrosarcoma cells in cyclic and continuous hydrostatic pressure (19); higher expression in nucleus pulposus than in articular cartilage of rat (22)
miR-563	FGF18 (fibroblast growth factor 18)	Possess broad mitogenic and cell survival activities, and are involved in embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion; plays a role in the maintenance of chondrocyte properties (23); stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis (24)
	HAS3 (hyaluronan synthase 3)	Involved in the synthesis of the unbranched glycosaminoglycan hyaluronan, or hyaluronic acid, which is a major constituent of the extracellular matrix;upregulated by IL-1 in bovine chondrocytes (25)
	IL7R (IL-7 receptor)	Blocking apoptosis during differentiation and activation of T lymphocytes; respond to IL-7 stimulation with increased production of matrix metalloproteinase-13 and with proteoglycan release from cartilage explants (26)
	GSPT1 (G1 to S phase transition 1)	
	FOXC2 (forkhead box C2)	The forkhead family of transcription factors which may play a role in the development of mesenchymal tissues; associated with the early stage of chondrogenic differentiation; regulated by BMP in skeletal precursor cells (27)
miR-603	Lyn (v-yes-1 Yamaguchi sarcoma viral related oncogene homolog)	A Src kinase gene which upon T-cell antigen receptor (TCR) stimulation, functions in the initial step of TCR-mediated signal transduction; inhibition by Src kinase inhibitor promotes chondrogenic gene expression and morphology in monolayer culture (28)
	MAX (MYC associated factor X)	A member of the basic helix-loop-helix leucine zipper family of transcription factors, which forms homodimers and heterodimers with Mad, Mxi1 and Myc; expressed mainly in the nucleus of proliferative chondrocytes and decreased as the chondrocytes mature (29)
	POR (P450 (cytochrome) oxidoreductase)	An endoplasmic reticulum membrane oxidoreductase which donate electrons directly from NADPH to all microsomal P450 enzymes; mutations in this gene have been associated with amenorrhea and disordered steroidogenesis, congenital adrenal hyperplasia and Antley-Bixler syndrome; specific inhibition leads to decreased cell proliferation and differentiation and induction of apoptosis in rat chondrocytes with decreased intracellular cholesterol content and Indian hedgehog expression (30)
	NKX3-1 (NK3 homeobox 1)	A transcription factor which is a putative prostate tumor suppressor expressed in a largely prostate-specific and androgen-regulated manner; act together with Sox9 to maintain the chondrogenic cell fate and promote early differentiation (31)
miR-766	PTGER2 (prostaglandin E receptor 2)	A receptor for prostaglandin E2; decreased by IL-1 in human chondrocytes (32); stimulation of human articular chondrocytes with PGE2 through the EP2 receptor suppresses proteoglycan accumulation and synthesis, aggrecan gene expression and decreases the type II collagen:type I collagen ratio; expressed at higher levels in knee cartilage than in ankle cartilage and in a OA grade-dependent manner (33); a specific EP2 agonist promotes regeneration of cartilage tissues with a physiological osteo-chondral boundary in rabbit injury models (34)
	SDC4 (Syndecan 4)	A transmembrane (type I) heparan sulfate proteoglycan that functions as a receptor in intracellular signaling; the mRNA level increase in cartilage tissue from heavily damaged area of human OA (35); specifically induced in type X collagen-producing chondrocytes both in human and in murine OA; knock-out mice are protected from cartilage damage in a surgically induced model of OA by decrease in ADAMTS-5 activity (36); interact with MMP-13 C-terminal domain in cultured human chondrocytes (37)
	SAT1 (spermidine/spermine N1-acetyltransferase 1)	A rate-limiting enzyme in the catabolic pathway of polyamine metabolism catalyzing the acetylation of spermidine and spermine, and involved in the regulation of the intracellular concentration of polyamines and their transport out of cells; increase in activity by parathyroid hormone (PTH) in rabbit costal chondrocytes (38)

1, a protein crosslinking actin filaments and regulating cytoskeletone rearrangement (51) were validated by RT-PCR (Fig. 2). IL-1 dose-dependently upregulated these 2 genes in articular chondrocytes. Lastly, the expression of miR-200a was examined in normal and OA cartilages from lesion and non-lesion area (Fig. 3). It was found that the expression of miR-200a was scant in the superficial zone compared to the deep zone in both normal and OA non-lesion cartilages. In OA lesion cartilage, the expression of miR-200a was downregulated.

Discussion

In this study, we identified miRNAs differentially regulated by IL-1β in human OA articular chondrocytes. By using integrative approach using simultaneous miRNA-mRNA profil-

Table 3. MiRNAs downregulated by IL-1β and their target mRNAs identified by integrative analysis

MicroRNA downregulated	mRNA upregulated	Comments
miR-181b	GPI (glucose phosphate isomerase)	Multifunctional phosphoglucose isomerase protein involved in energy pathways; implicated as an autoantigen in rheumatoid arthritis; conformational changes under acidic conditions, such as those encountered in the synovial fluid of arthritic joints, results in increased association and deposition of fibronectin fibrils on the joint surface (39)
	GSTA-4 (glutathione S-transferase alpha 4)	Involved in cellular defense against toxic, carcinogenic, and pharmacologically active electrophilic compounds; ablation augments 4-hydroxynonenal cytotoxicity in human OA chondrocytes (40)
	P4HA2 (prolyl 4-hydroxylase, alpha polypeptide II)	A key enzyme in collagen synthesis which catalyzes the formation of 4-hydroxyproline essential to the proper three-dimensional folding of newly synthesized procollagen chains;exposure of primary human articular chondrocytes to 1% oxygen increase transcript levels, while treatment with 2-methoxyestradiol reduce transcriptional activity (41)
	PTHR1 (parathyroid hormone receptor 1)	A receptor for parathyroid hormone(PTH) and for parathyroid hormone-like hormone; expressed principally by proliferative and prehypertrophic chondrocytes in endochondral growth (42); increase by co-culture of human articular chondrocytes with non-sclerotic subchondral osteoblast (43); activation of PTH/PTHrP receptor signaling by hPTH results in the inhibition of chondrogenic differentiation in full-thickness articular cartilage defects (44); delays chondrocyte hypertrophy mediated by both Runx2-dependent and -independent mechanisms (45)
	MAP3K6 (mitogen-activated protein kinase kinase kinase 6)	A member of the serine/threonine protein kinase family; weakly activate MAPK7, but not MAPK1/ERK or MAPK14
	SLC26A11 (solute carrier family 26, member 11)	A sodium independent sulfate transporter; a member of the solute linked carrier 26 family of anion exchangers;essential for homeostasis and intracellular electrolyte balance; expressed in bovine articular chondrocytes, and regulates sulfate uptake, an essential step in the pathway for sulphation of glycosaminoglycans (46)
miR-200a	RBL2 (retinoblastoma-like 2 (p130))	Contributes to cell cycle exit, and knock out leads to deregulated proliferation, reduced expression of Cbfa1, and reduced hypertrophic differentiation of chondrocytes (47); effectors of FGF-mediated growth inhibition in chondrocytes (48)
	STMN1 (stathmin 1)	A ubiquitous cytosolic phosphoprotein, involved in the regulation of the microtubule filament system by destabilizing microtubules; proliferating chondrocytes in vivo exhibit higher level of stathmin than either resting or growth zone in the growth plate (49)
	PBX1 (pre-B-cell leukemia homeobox 1)	A component of various protein complexes implicated in developmental gene expression; knock-out leads to embryonic death with widespread patterning defects of the axial and appendicular skeleton, decrease in chondrocyte proliferation, and an increase in hypertrophic chondrocytes (50)
	PTHR1	
	TEN (Tensin 1)	Localizes to focal adhesions and crosslinks actin filaments; a substrate of calpain II; upregulated by BMP in bovine and murine chondrocytes (51)
	Tbx15 (T-box-15)	A phylogenetically conserved family of transcription factors that regulate a variety of developmental processes; expressed during limb development in the early limb bud, in prehypertrophic chondrocytes of cartilaginous templates, and in mesenchymal precursor cells and prehypertrophic chondrocytes of the vertebral column and the head of mouse embryo; mutants display a general reduction of bone size, changes of bone shape, reduction in the size of cartilaginous templates, and reduction in proliferation of prehypertrophic chondrocytes (52)

Table 3. Continued

MicroRNA downregulated	mRNA upregulated	Comments
miR-31	HTRA1 (HtrA serine peptidase 1)	Member of the trypsin family of serine proteases; regulates the availability of insulin-like growth factors (IGFs) by cleaving IGF-binding proteins; increased in the knee and TM joints of mouse OA models in early stages of the disease (53); HtrA1-generated aggrecan fragments containing the VQTV (356) neoepitope were significantly more abundant in osteoarthritic cartilage compared with cartilage from healthy joints (54)
	FGFR3 (fibroblast growth factor receptor 3)	Binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance; mutation leads to achondroplasia; induces premature senescence in chondrocytes, manifested as growth arrest, alteration of cellular shape, and loss of the extracellular matrix (55)
	CYTL1 (cytokine-like 1)	Expressed in bone marrow and cord blood mononuclear cells; regulates chondrogenesis as a novel autocrine factor, but not hypertrophic maturation of chondrocytes during cartilage development (56)
	HOXA2 (homeobox A2)	Encodes a DNA-binding transcription factor which may regulate morphogenesis, and differentiation; the encoded protein may be involved in the placement of hindbrain segments in the proper location along the anterior-posterior axis during development; persistent expression in chondrogenic cells results in overall chondrodysplasia with delayed cartilage hypertrophy, mineralization, and ossification in mouse embryo (57)
	GPX7 (glutathione peroxidase 7)	An antioxidant enzyme that disproportionate reactive oxygen species; increased by IL-1 and IL-6 dose and time-dependently in bovine chondrocytes (58)
	CDK4 (cyclin-dependent kinase 4)	A catalytic subunit of the protein kinase complex important for cell cycle G1 phase progression; overexpression leads to resistance to FGF-induced p107 dephosphorylation and growth arrest (59)
miR-565	ENG (endoglin)	A major glycoprotein of the vascular endothelium,and a component of the transforming growth factor (TGF) beta receptor complex; mutations in this gene cause hereditary hemorrhagic telangiectasia; expressed on human chondrocytes at levels comparable with endothelial cells and forms higher order complexes with the type I and II TGFβ receptors (60)
	BARX1 (BARX homeobox 1)	A member of the Bar subclass of homeobox transcription factors which play a role in developing teeth and craniofacial mesenchyme of neural crest origin; expressed in the developing joint and articular cartilage and has an inhibitory effect on chondrogenic initiation (61)
	CTSK (cathepsin K)	A lysosomal cysteine proteinase involved in bone remodeling and resorption; induced in phenotypically altered chondrocytes in human OA and animal OA model (62,63); chemical inhibitor results in mild to moderate beneficial effects on gross and histopathological parameters of OA and reduction of biomarkers of collagen type I and II degradation in the canine partial medial meniscectomy model of OA (64)

ing and bioinformatic analysis, we further attempted to explore the miRNA target with more precision.

Precise identification of miRNA targets is important for elucidation of the pathogenesis of human diseases resulting from miRNA misregulation. Although miRNAs are believed to regulate their targets primarily through inhibition of translation, increasing evidence shows that miRNAs also influence the amount of their target mRNAs. In both mammalian and Drosophila systems, miRNAs have been shown to accelerate target mRNA degradation through the pathway of deadenylation, consequently decreasing target mRNA abundance (5). The rules that govern miRNA target specificity remain elusive. Several computational methods have been developed to predict mRNAs targeted by miRNAs in animals. One of such methods is TargetScan, which was applied to predict miRNA target sites conserved among orthologous 3' UTRs of vertebrates (65). When using 3' UTR regions of human, mouse, and rat, 451 human targets were predicted by TargetScan using score and rank cutoffs, but it gave as many as 31% false-positive predictions (65). The fact that miRNAs cause degradation of their targets and that a large number of mRNAs are regulated in this way leads to the prediction that a miRNA expressed at a high or low level in a specific cell or tissue might leave a signature on the mRNA expression profile (4) In previous reports, predicted target genes of known tissue-specific miRNAs, such as miR-122 in liver, miR-1 in heart/skeletal muscle and miR-7 in pituitary, were expressed at significantly lower levels in their cognate tissue relative to all other tissues, corroborating the usefulness of miRNA-mRNA integrative analysis (66,67). Our integrative miRNA-mRNA analysis in chondrocytes, thus, potentially provide in vitro evidence of gene regulation in addition to the in silico evidence provided by the sequence-based algorithms.

The physiological and pathogenetic role of miRNAs in the

maintenance of joint homeostasis and the development of arthritis is recently being elucidated. Dicer, a component for biogenesis of miRNAs, was found to have an essential function for skeletal development, with dicer-null growth plates showing a progressive reduction in the proliferating pool of chondrocytes, leading to severe skeletal growth defects and premature death of mice (68). In rheumatoid arthritis (RA), miR-155 was found to be overexpressed in synovial fibroblasts and this correlated with the reduced expression of MMP-3 suggesting involvement of miRNAs in the regulation of inflammation and joint destruction (69). MiR-146a is induced by a variety of microbial components and pro-inflammatory cytokines, such as IFN-α, IL-1β and TNFα (70). MiR-146a has been gaining interest as it was found to serve as a regulator of key signaling intermediates of the pro-inflammatory Toll-like receptor (TLR)-MyD88 pathway including IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) (71). MiR-146a was

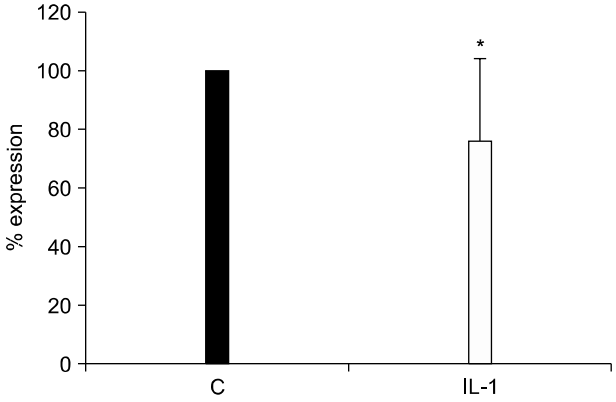

Figure 1. Real-time RT-PCR results for miR-200a in chondrocytes in response to IL-1β. Total RNA was isolated from IL-1β treated monolayer chondrocytes obtained from donors different from those that were used in miRNA microarray. The RNA, U6 small nuclear 2 (RNU6-2) gene was used as a control to normalize differences in total RNA levels in each sample. The value of each control sample was set at 100 and was used to calculate the fold change in IL-1β treated chondrocytes. Data are from triplicate experiments using chondrocytes from 3 different donors. *denoted $p<0.05$ compared to control by using Mann-Whitney U test.

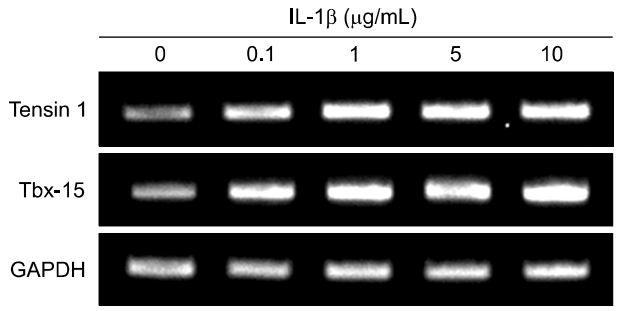

Figure 2. RT-PCR results for tensin-1 and Tbx-15 in chondrocytes in response to IL-1β. Total RNA was isolated from IL-1β treated monolayer chondrocytes obtained from donors different from those that were used in mRNA microarray. GAPDH gene was used as a control to normalize differences in total RNA levels in each sample. Data are representative of duplicate experiments using chondrocytes from 3 different donors.

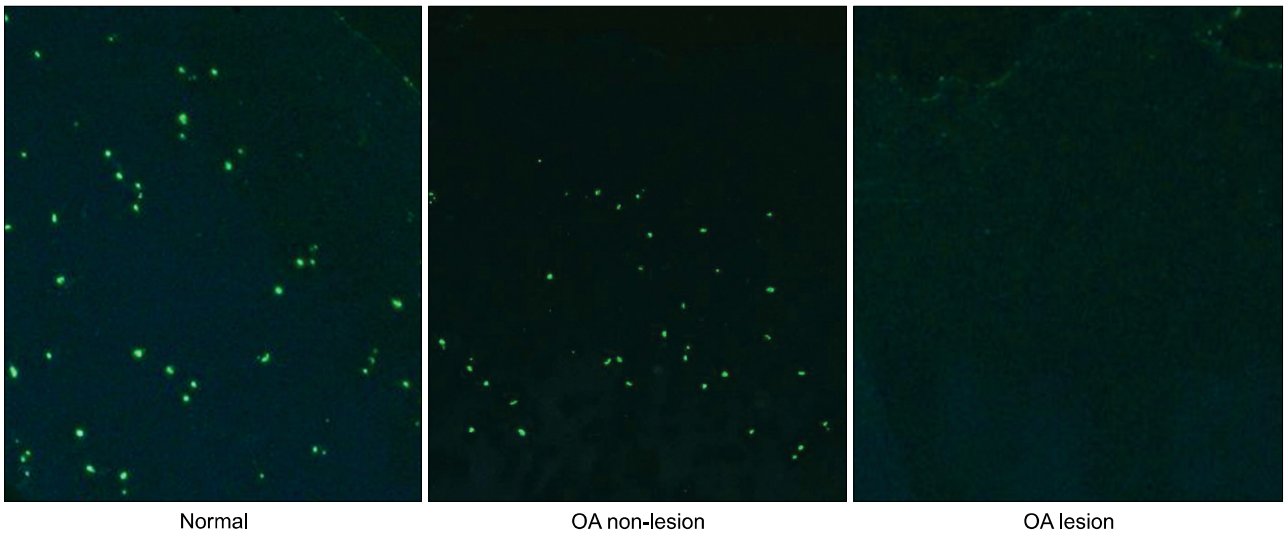

Figure 3. In situ hybridization for miR-200a in normal and OA cartilages. Cartilage tissues obtained from OA patients at the time of joint replacement surgery were obtained from both lesion and non-lesion areas (defined as areas with preserved cartilage surface and thickness). For normal cartilage, macro and microscopically normal cartilage samples obtained from the femoral head of patients with femoral neck fracture were used. Data are representative of specimens from 4 different donors each for normal and OA (×100, original magnification).

overexpressed and its expression was induced by stimulation with TNF-α and IL-1β in RA synovium (72). MiR-146a was also found to be expressed in OA cartilage (73). The expression of miR-200a has not previously been reported in chondrocytes. Previous studies have shown that miR-200a plays a role in inhibition of tumorigenesis by inhibiting epithelial-to-mesenchymal transition, an initial step in tumorigenesis (11,19,74). In addition, miR-200a regulate Wnt/β-catenin signaling by directly targeting the 3' UTR of β-catenin mRNA, making it the first miRNA involved in the regulation of β-catenin levels (75). In our study, β-catenin was not identified as miR-200a target, which might have resulted from the limitation in microarray technique or difference in stimulating condition. Considering the role of Wnt signaling components in the maintenance and destruction of articular cartilage, investigation on the pathogenetic role of miR-200a in OA would be an interesting subject. In our in situ hybridization, miR-200a was downregulated in OA lesion cartilages compared to normal or OA non-lesion cartilage. This distribution is reciprocal of that of β catenin, further raising the issue of miR-200a-β-catenin regulation (76).

Recent publications have reported the correlation of MMP-13, the most potent MMP implicated in the degradation of cartilage matrix, with specific miRNAs (77-80). In our study, mRNA microarray analysis revealed upregulation of MMP-13, MMP-3 and MMP 1 after 4 hours of IL-1β stimulation as expected(data not shown), however, we could not identify relevant miRNAs that regulate MMPs. Considering that the degree of differential expression of miRNA was modest (less than 2 fold), it is speculated that regulation of RNA degradation by miRNA may not be prominent under the condition of our experiment (1 ng/μL of IL-1β for 4 hours). In a report by Akhtar et al., miRNA different from ours was identified after 6 hours of 10 ng/μL IL-1β stimulation of OA chondrocytes, most of which were downregulated, thus treatment condition may have profound effect on the expression and regulation of miRNA in chondrocytes.

Our study has other limitations. It has been shown that some miRNAs regulate gene expression only at the protein level, and intergration of miRNA microarray and proteomic analysis would be more relevant in these cases. For example, microarray analysis of Dicer-null chondrocytes showed limited expression changes in miRNA-target genes, suggesting that chondrocytic miRNAs do not directly regulate target RNA abundance. Although it is difficult to validate miRNA target predictions from effects on translation due to the technical difficulties of genome-wide quantitative protein profiling, one report integrated genetic, bioinformatic and proteomic approaches in an attempt to identify new genes and their collaborative networks involved in OA pathogenesis (77). Integration of miRNA and proteomic data with miRNA gene-target prediction algorithms generated a potential "interactome" network consisting of 11 miRNAs and 58 proteins linked by 414 potential functional associations, suggesting the feasibility of such an approach.

Conclusion

miRNAs regulated by IL-1β were identified in human articular chondrocytes along with mRNAs they regulate by using integrative miRNA-mRNA analysis. MiR-200a were found to be expressed in human OA and normal cartilages, with downregulation in OA lesion cartilages. It is suggested that miRNAs may play a role in the regulation of cartilage degradation in OA.

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