Eur J Nutr
DOI 10.1007/s00394-016-1375-x

ORIGINAL CONTRIBUTION

Epigallocatechin-3-*O*-gallate modulates global microRNA expression in interleukin-1β-stimulated human osteoarthritis chondrocytes: potential role of EGCG on negative co-regulation of microRNA-140-3p and ADAMTS5

Zafar Rasheed[1] · Naila Rasheed[1] · Osama Al-Shaya[2]

Received: 27 August 2016 / Accepted: 22 December 2016

Abstract
Purpose MicroRNAs (miRNAs) are short, non-coding RNAs involved in almost all cellular processes. Epigallocatechin-3-O-gallate (EGCG) is a green tea polyphenol and is known to exert anti-arthritic effects by inhibiting genes associated with osteoarthritis (OA). This study was undertaken to investigate the global effect of EGCG on interleukin-1β (IL-1β)-induced expression of miRNAs in human chondrocytes.
Methods Human chondrocytes were derived from OA cartilage and then treated with EGCG and IL-1β. Human miRNA microarray technology was used to determine the expression profile of 1347 miRNAs. Microarray results were verified by taqman assays and transfection of chondrocytes with miRNA inhibitors.
Results Out of 1347 miRNAs, EGCG up-regulated expression of 19 miRNAs and down-regulated expression of 17 miRNAs, whereas expression of 1311 miRNAs remains unchanged in IL-1β-stimulated human OA chondrocytes. Bioinformatics approach showed that 3`UTR of ADAMTS5 mRNA contains the 'seed-matched-sequence' for hsa-miR-140-3p. IL-1β-induced expression of ADAMTS5 correlated with down-regulation of hsa-miR-140-3p. Importantly, EGCG inhibited IL-1β-induced ADAMTS5 expression and up-regulated the expression of hsa-miR-140-3p. This EGCG-induced co-regulation between ADAMTS5 and hsa-miR-140-3p becomes reversed in OA chondrocytes transfected with anti-miR-140-3p.
Conclusions This study provides an important insight into the molecular basis of the reported anti-arthritic effects of EGCG. Our data indicate that the potential of EGCG in OA chondrocytes may be related to its ability to globally inhibit inflammatory response via modulation of miRNAs expressions.

Keywords Osteoarthritis · MicroRNAs · Chondrocytes · Microarray · Hsa-miR-140-3p · ADAMTS5

Introduction

Osteoarthritis (OA) is a most common chronic degenerative joints' disorder characterized by progressive damage of articular cartilage resulting pain and disability. Proteolytic degradation of articular cartilage is a key pathological event in OA [1]. A major component of the cartilage extracellular matrix is aggrecan, which is basically a proteoglycan that imparts compressive resistance to the articular tissues [1, 2]. In an early stage of OA, aggrecan degradation by aggrecanases is a most frequent event for disease progression [3]. In cartilage, two types of aggrecanases (or a disintegrin and metalloprotease with thrombospondindomains; ADAMTS) are present; aggrecanase-1 (or ADAMTS4) and aggrecanse-2 (or ADAMTS5) [1–3]. Studies have shown that ADAMTS5 knockout mice were protected from early cartilage degradation in OA animal models [4]; however, this protection was not found in ADAMTS4 knockout mice [5]. Therefore, ADAMTS5 seems to be an obvious therapeutic target for OA therapy.

✉ Zafar Rasheed
zafarrasheed@qumed.edu.sa

[1] Department of Medical Biochemistry, College of Medicine, Qassim University, P.O. Box 6655, Buraidah 51452, Saudi Arabia

[2] Department of Orthopedics, King Fahd Medical City, Riyadh, Saudi Arabia

Published online: 21 January 2017

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs having considerable roles in post-transcriptional regulation of gene expressions [6]. Currently, 1881 precursors of human miRNA and 2588 mature human miRNAs have been registered in the latest miRBase database (release 21, June 2014, http://www.miRBase.org). Importantly, it is estimated that more than 60% of all human protein-coding genes are seem to maintain pairing with miRNAs [7, 8]. In OA, alterations in miRNAs' expression have been reported in cartilage pathophysiology and cartilage homeostasis [9]. Studies have shown that cartilage-specific dicer-knockout mice showed severe skeletal growth defects or premature death [10]. Moreover, Miyaki et al. showed mice deficient with miRNA-140 developed age-related OA symptoms [11], which clearly suggested important role of miRNAs in OA progression. Recently, we also demonstrated the IL-1β-induced global miRNAs expression in human OA chondrocytes. Our results identified several novel miRNAs, which have been targeted to the most common OA-relevant genes [12]. In another study, we showed that miRNA-27b is a direct regulator of matrix metalloproteinase-13 (MMP-13) in human OA chondrocytes [13]. Furthermore, we also demonstrated that hsa-miR-26a-5p regulates the expression of inducible nitric oxide synthase (iNOS) via activation of nuclear transcription factor (NF)-κB in stimulated human OA chondrocytes [14]. In addition, other miRNAs, including miR-320, miR-381, miR-9, miR-602, miR-608, miR-127-5p, and miR-558, have also been recently demonstrated and found to have significant role in regulation of genes relevant to OA pathogenesis [15–20]. These findings clearly indicated the significance of miRNAs in cartilage homeostasis as their deregulation strongly correlated with the onset of disease.

Epigallocatechin-3-O-gallate (EGCG) is a most abundant polyphenol of green tea, and its potential value as an anti-arthritic/anti-inflammatory agent has been demonstrated in several human and animal investigations [21, 22]. In the past decade, Haqqi and colleagues performed extensive studies on EGCG and proved that EGCG has cartilage-preserving and chondroprotective activity [22–27]. In recent years, several studies revealed that EGCG has potential to modulate miRNAs expression in various patients [28–32]. Recently, we determined the role of EGCG on miRNA regulation for the first time in human OA chondrocytes. Our novel results showed that EGCG inhibited cyclooxygenase (COX)-2 expression via up-regulation of hsa-miR-199a-3p expression in stimulated human OA chondrocytes [33]. In the present study, we demonstrated for the first time the potential role of EGCG on global miRNA modulation in IL-1β-stimulated human OA chondrocytes. Our results identified several novel OA-relevant miRNAs, which have been targeted and regulated by EGCG. These novel pharmacological actions of EGCG on miRNA expression may be of value in the design of novel therapies for OA and other joint disorders.

Methods

Discarded osteoarthritic cartilage and culture of chondrocytes

The present study has been carried out in accordance with Code of Ethics of World Medical Association (Declaration of Helsinki as revised in Tokyo 2004) for humans and was approved by King Fahd Medical City, KSA (IRB Registration # with KACST, KSA: H-01-R-012; IRB Registration # with OHRP/NIH, USA:IRB00008644; Approval Number Federal Wide Assurance NIH, USA: FWA00018774). With Institutional Review Board (IRB) approval, discarded cartilages were obtained from the knee joints of OA patients undergoing joint replacement surgery. The cartilage with smooth articular surface was resected and digested by pronase and collagenase (Roche Diagnostics, Mannheim, Germany) treatment as described previously [34]. Isolated chondrocytes were plated at a density of 1.2×10^6/ml in six well tissue culture dishes (Millipore, Darmstadt, Germany) in complete DMEM medium as previously described [35].

Treatment of primary human OA chondrocytes with IL-1β and EGCG

Human OA chondrocytes (1.2×10^6/ml) were plated in complete DMEM medium (catalog # SLM-120-B, Millipore) and were serum-starved for 12 h/overnight. Starved human chondrocytes were pretreated with EGCG (20–50 μM) (purity ≥95%, Calbiochem, CA, USA) for 2 h prior to stimulation with IL-1β (5 ng/ml; catalog # IL038, EMD Millipore corporation, Temecula, CA, USA) for 24 h as described previously [27, 36]. OA chondrocytes cultured without IL-1β or EGCG served as experimental controls. After 24 h of IL-1β or EGCG treatment, viability of chondrocytes was examined by Cell Titer-Glo Luminescent Cell Viability Assay kit (catalog # G7573, Promega, Wl, USA).

Preparation of microRNAs for microRNA microarray analysis

Total RNA (containing microRNA fraction) from treated or untreated human OA chondrocytes was prepared using mirVana microRNA isolation kit (catalog # AM1560, Ambion, CA, USA) as described by manufacturer instructions. MicroRNA microarray analysis was performed using the miRNA Complete Labeling and Hybridization kit (Agilent Technologies, Santa Clara, CA, USA) as described previously [12]. The processed samples were loaded onto

the human microRNA Microarray Release 16.0, 8×60 k microarrays, of which 1347 human miRNAs were represented based on miRBase 16 (G2534-60014, Agilent Technologies) and the hybridized images were scanned using a G2505C Agilent Microarray Scanner (scan control version A.8.4.1, Agilent Technology).

Bioinformatics approach for microRNA target prediction

Target Scan bioinformatics algorithm (http://www.targetscan.org/) was used to analyze miRNA target prediction. This web-based computational tool applied different algorithms, based on several parameters calculated individually for each miRNA to identify the predicted mRNA as described previously [12, 37].

Reverse transcription and quantitative RT-PCR analysis

Total RNA (0.5–1.0 µg) was reverse-transcribed using SuperScript First Strand cDNA synthesis kit (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. The expression of ADAMTS5 mRNA and hsa-miR-140-3p was quantified using TaqMan Gene Expression Assays (Applied Biosystems). GAPDH/RNU6B expression was used as an endogenous control. Real-time PCR amplification and data capture were carried out using the Step One Real-Time PCR System (Applied Biosystems) as described previously [14]. Relative expression levels were analyzed using ΔΔCT method [38].

Transfection of chondrocytes with anti-miRNAs

Human OA chondrocytes were transfected with anti-miRNAs (100 nM; Ambion/Qiagen) at a 100 nM concentration, using the calcium phosphate precipitation method as described previously [14, 33, 39]. Following transfection, cells were treated with EGCG or IL-1β for 24 h to analyze the expression of miRNA and mRNA as previously described [14, 33].

Statistical analysis

Statistical comparisons were performed by One-way ANOVA analysis followed by Tukey's post-hoc analysis or Two-way ANOVA followed by Bonferroni post-hoc tests using Graph Pad Prism-5 (San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

EGCG was non-toxic to human OA chondrocytes *in-vitro*

In these studies, pretreatment of human OA chondrocytes for 24 h with varying concentration of EGCG (up to 50 µM) was found to be non-toxic and showed no effect on the cell viability (results not shown). Based on these results, the maximum concentration of EGCG used in these studies was 50 µM.

EGCG-induced microRNA profiling in IL-1β-stimulated human OA primary chondrocytes

To assess the putative role of EGCG-induced miRNAs modulation in OA cartilage pathology, these studies were performed. Purity of isolated RNA fractions was assessed from A260/A280 ratio and was found to be greater than 1.9 for all isolated samples. Out of 1347 miRNAs immobilized on the microarray, only 36 miRNAs (with a fold-change cutoff ≥1.0) were differentially expressed upon EGCG or IL-1β treated human OA chondrocytes. Figure 1 shows the results of the three-way hierarchical clustering of miRNAs. Each row represents an miRNA, and each column represents samples: (1) unstimulated (controls) OA chondrocytes; (2) IL-1β-stimulated OA chondrocytes; and (3) EGCG pretreated and then IL-1β-stimulated OA chondrocytes. The color scale at the bottom illustrated the relative expression level of an miRNA across all samples. Amongst these 36 differentially expressed human miRNAs, 19 were up-regulated and 17 were down-regulated by EGCG in IL-1β-stimulated human OA chondrocytes. The complete characterization of EGCG-induced up- or down- regulations of differentially expressed miRNAs has been summarized in Tables 1 and 2, respectively. Figures 2 and 3 show fold change of EGCG-induced up-regulated and down-regulated miRNAs in IL-1β-stimulated human OA chondrocytes, respectively.

Bioinformatics prediction of hsa-miRNA-140-3p targeting 3`UTR of ADAMTS5 mRNA (NM_007038.4)

Target Scan algorithm identified the sequence conserved in the 3`UTR of ADAMTS5 mRNA complementary to the hsa-miR-140-3p seed sequence. Not only in human, sequence at 3`UTR of ADAMTS5 mRNA also predicted in other species. The complete details of Target Scan prediction have been shown in Fig. 4.

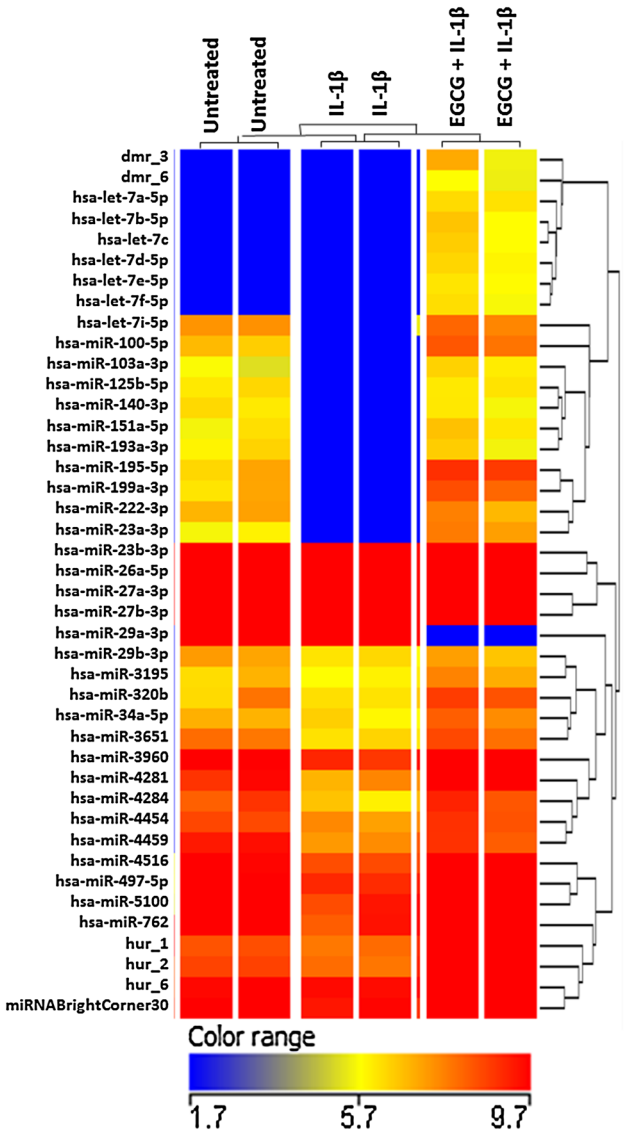


Fig. 1 EGCG modulated global microRNA expression. Heat map diagram and hierarchical clustering of differentially expressed miRNAs in human OA chondrocytes treated with EGCG and IL-1β. Each row represents an miRNA expression, and each column represents a sample of untreated OA chondrocytes (Untreated), OA chondrocytes treated with IL-1β alone (IL-1β), and OA chondrocytes treated with EGCG and IL-1β (EGCG + IL-1β). The *color scale* shown at the *bottom* illustrates the relative expression level of an miRNA across all samples

Inverse correlation between hsa-miR-140-3p and ADAMTS5 expressions

An inverse correlation between miR-140-3p expression and ADAMTS5 expression was observed in IL-1β-stimulated human OA chondrocytes. Stimulation of human OA primary chondrocytes with 24 h IL-1β treatment showed a significant down-regulation of miR-140-3p expression (Fig. 5a, $p < 0.05$) and significant up-regulation of ADAMTS5 (Fig. 5b, $p < 0.001$). This inverse correlation was verified by transfection of OA chondrocytes with anti-miR-140-3p (100 nM) and was then either stimulated or not with IL-1β (5 ng/ml). Quantitative RT-PCR results showed that human OA chondrocytes transfected with anti-miR-140-3p and then stimulated with IL-1β, significantly inhibited miR-140-3p expression as compared with unstimulated chondrocytes ($p < 0.001$). OA chondrocytes stimulated with IL-1β alone or transfected chondrocytes without IL-1β stimulation were used as experimental controls (Fig. 5c). Furthermore, transfection of OA chondrocytes with anti-miR-140-3p showed marked increased of IL-1β-induced ADAMTS-5 mRNA expression as compared with unstimulated OA chondrocytes ($p < 0.0001$). Human OA chondrocytes stimulated with IL-1β alone or anti-miR-140-3p-transfected chondrocytes without stimulation were used as experimental controls (Fig. 5d).

EGCG up-regulated hsa-miR-140-3p expression and down-regulated ADAMTS5 expression

Stimulation of human OA primary chondrocytes with IL-1β alone showed a significant down-regulation of hsa-miR-140-3p expression ($p < 0.05$). Interestingly, pretreatment with EGCG significantly enhanced IL-1β-induced hsa-miR-140-3p expression in a dose-dependent manner (Fig. 6a, $p < 0.05$). However, EGCG alone had no significant effect on hsa-miR-140-3p expression ($p > 0.05$). Figure 6b showed human OA chondrocytes treated with IL-1β alone had higher level of ADAMTS5 mRNA compared with untreated OA chondrocytes ($p < 0.001$). However, pretreatment of chondrocytes with EGCG showed marked declined of IL-1β-induced ADAMTS5 mRNA expression in a dose-dependent manner ($p < 0.05$). As expected, treatment of OA chondrocytes with EGCG alone showed no effect on ADAMTS5 mRNA expression ($p > 0.05$).

EGCG-induced negative regulation between hsa-miR-140-3p and ADAMTS5

Involvement of EGCG in the negative co-regulation between hsa-miR-140-3p and ADAMTS5 was confirmed by transfection of human OA chondrocytes with anti-miR-140-3p. Transfection of OA chondrocytes with anti-miR-140-3p synergize with IL1-β in reducing miR-140-3p levels (Fig. 6c, $p < 0.05$). Whereas, EGCG treatment remarkably and consistently up-regulated the IL-1β-inhibited hsa-miR-140-3p expression in a dose-dependent manner (Fig. 6c, $p < 0.05$). Under identical experimental conditions, ADAMTS5 mRNA expression was also quantified, and results showed that human OA chondrocytes treated with IL-1β alone had a higher level of ADAMTS5 mRNA compared to untreated OA chondrocytes ($p < 0.01$).

Table 1 EGCG up-regulated microRNAs in IL-1β-stimulated human osteoarthritis chondrocytes

Systematic name	Mirbase accession ID	Active sequence	EGCG + IL-1β vs. IL-1β alone		Control vs. IL-1β		EGCG-induced up-regulation
			Fold change		Fold change		Net fold change
			Mean	±SD	Mean	±SD	
hsa-let-7a-5p	MIMAT0000062	AACTATACAACCTACTACCT	−2.83	4.58	−3.43	0.04	0.60
hsa-let-7b-5p	MIMAT0000063	AACCACACAACCTACTACC	−1.44	1.85	−1.88	0.58	0.44
hsa-let-7c	MIMAT0000064	AACCATACAACCTACTACC	−0.21	1.63	−1.94	0.39	1.73
hsa-let-7d-5p	MIMAT0000065	AACTATGCAACCTACTACC	−42.47	47.88	−59	1.4×10^{-5}	16.53
hsa-let-7f-5p	MIMAT0000066	AACTATACAATCTACTACCTC	−1.81	2.28	−2.83	0.56	1.02
hsa-let-7i-5p	MIMAT0000415	AACAGCACAAACTACTACCTC	−0.32	2.06	−1.91	0.67	1.59
hsa-miR-100-5p	MIMAT0000098	CACAAGTTCGGATCTACGG	−1.99	2.76	−3.3	1.87	1.31
hsa-miR-140-3p	MIMAT0004597	CCGTGGTTCTACCCT	−1.22	1.59	−2.08	0.54	0.86
hsa-miR-193a-3p	MIMAT0000459	ACTGGGACTTTGTAGGC	−35.1	39.37	−73.39	7.6×10^{-6}	38.29
hsa-miR-199a-3p	MIMAT0000232	TAACCAATGTGCAGACTACT	−66.03	113.54	−98.57	102.61	32.54
hsa-miR-27b-3p	MIMAT0000419	GCAGAACTTAGCCACTGT	−37.5	88.33	−53.73	1.4×10^{-5}	16.23
hsa-miR-29a-3p	MIMAT0000086	TAACCGATTTCAGATGGTGC	0.06	1.93	−2.86	0.08	2.92
hsa-miR-320b	MIMAT0005792	TTGCCCTCTCAACCC	−1.17	1.67	−1.41	0.15	0.24
hsa-miR-34a-5p	MIMAT0000255	ACAACCAGCTAAGACACTGC	−28.88	32.19	−72.69	3.2×10^{-5}	43.81
hsa-miR-3960	MIMAT0019337	CCCCCGCCTCCG	−0.4	2.03	−1.02	0.01	0.62
hsa-miR-4284	MIMAT0016915	CCCCCCTCCCCG	−32.89	36.83	−70.96	2.7×10^{-5}	38.07
hsa-miR-4454	MIMAT0018976	ATGGGGTGATGTGAGC	−0.27	3.26	−2.29	0.49	2.02
hsa-miR-497-5p	MIMAT0002820	ACAAACCACAGTGTGCTG	−37.76	42.45	−43.35	2.2×10^{-5}	5.59
hsa-miR-5100	MIMAT0022259	AGAGGCACCGCTGG	−0.25	4.5	−2.83	1.10	2.58

Table 2 EGCG down-regulated microRNAs in IL-1β-stimulated human osteoarthritis chondrocytes

Systematic name	Mirbase accession ID	Active sequence	EGCG + IL-1β vs. IL-1β alone		Control vs. IL-1β		EGCG-induced down-regulation
			Fold change		Fold change		Net fold change
			Mean	±SD	Mean	±SD	
hsa-let-7e-5p	MIMAT0000066	AACTATACAACCTCCTACC	−1.71	2.24	−1.64	0.82	−0.07
hsa-miR-103a-3p	MIMAT0000101	TCATAGCCCTGTACAATG	−39.08	43.98	−1	0.00	−38.08
hsa-miR-125b-5p	MIMAT0000423	TCACAAGTTAGGGTCTC	−238.91	274.72	−107.11	0.00	−131.8
hsa-miR-151a-5p	MIMAT0004697	ACTAGACTGTGAGCTCC	−35.12	39.40	−1	0.00	−34.12
hsa-miR-195-5p	MIMAT0000461	GCCAATATTTCTGTGCTGC	−2.64	1.66	−1.4	0.39	−1.24
hsa-miR-222-3p	MIMAT0000279	ACCCAGTAGCCAG	−31.42	35.13	−1	0.00	−30.42
hsa-miR-23a-3p	MIMAT0000078	GGAAATCCCTGGCAATGT	−79.63	136.16	−57.37	59.69	−22.26
hsa-miR-23b-3p	MIMAT0000418	GGTAATCCCTGGCAATG	−159.25	182.73	−99.19	2.7×10^{-5}	−60.06
hsa-miR-26a-5p	MIMAT0000082	AGCCTATCCTGGATT	−37.00	41.57	−1	0.00	−36.00
hsa-miR-27a-3p	MIMAT0000084	GCGGAACTTAGCCACTG	−141.29	104.26	−131.28	2.1×10^{-5}	−10.01
hsa-miR-29b-3p	MIMAT0000100	AACACTGATTTCAAATGGTGC	−35.52	39.86	−1	0.00	−34.52
hsa-miR-3195	MIMAT0015079	AACCCGGGCCCG	−37.95	42.66	−1	0.00	−36.95
hsa-miR-3651	MIMAT0018071	TCATGTACCAGCGACC	−24.46	27.09	−1	0.00	−23.46
hsa-miR-4281	MIMAT0016907	CCCCCCTCCCCG	−1.31	1.88	−1.3	0.19	−0.01
hsa-miR-4459	MIMAT0018981	CTCCACCTCCTCCG	−3.84	2.20	0.09	1.58	−3.93
hsa-miR-4516	MIMAT0019053	GCCCCGACCCTTC	−6.87	5.11	−1.14	0.03	−5.73
hsa-miR-762	MIMAT0010313	GCTCGGCCCCGG	−30.94	34.57	−1	0.00	−29.94

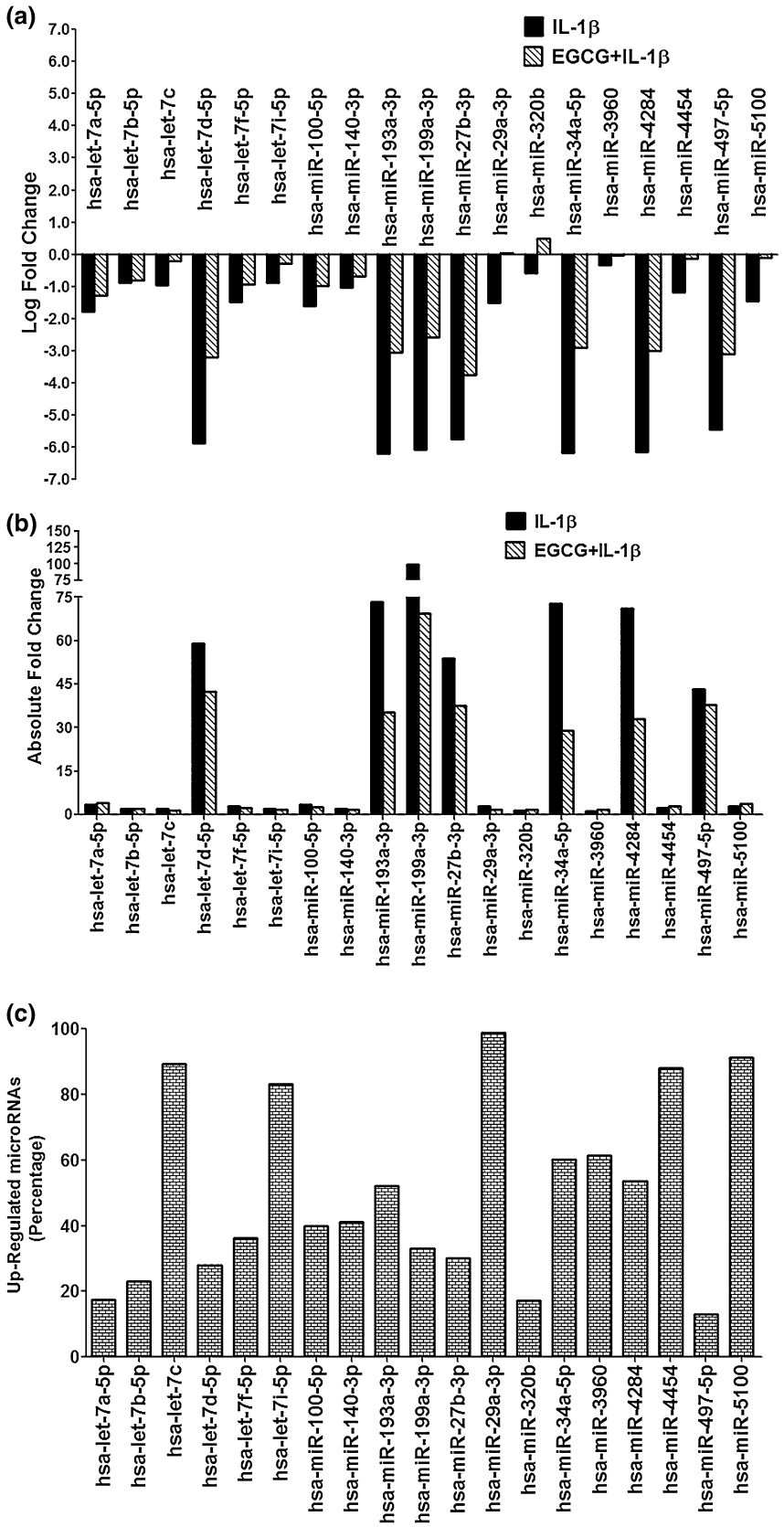


Fig. 2 EGCG up-regulated microRNAs. EGCG-induced up-regulated miRNAs in log fold change (**a**), absolute fold change (**b**), and percent fold change (**c**) in IL-1β-stimulated human OA chondrocytes

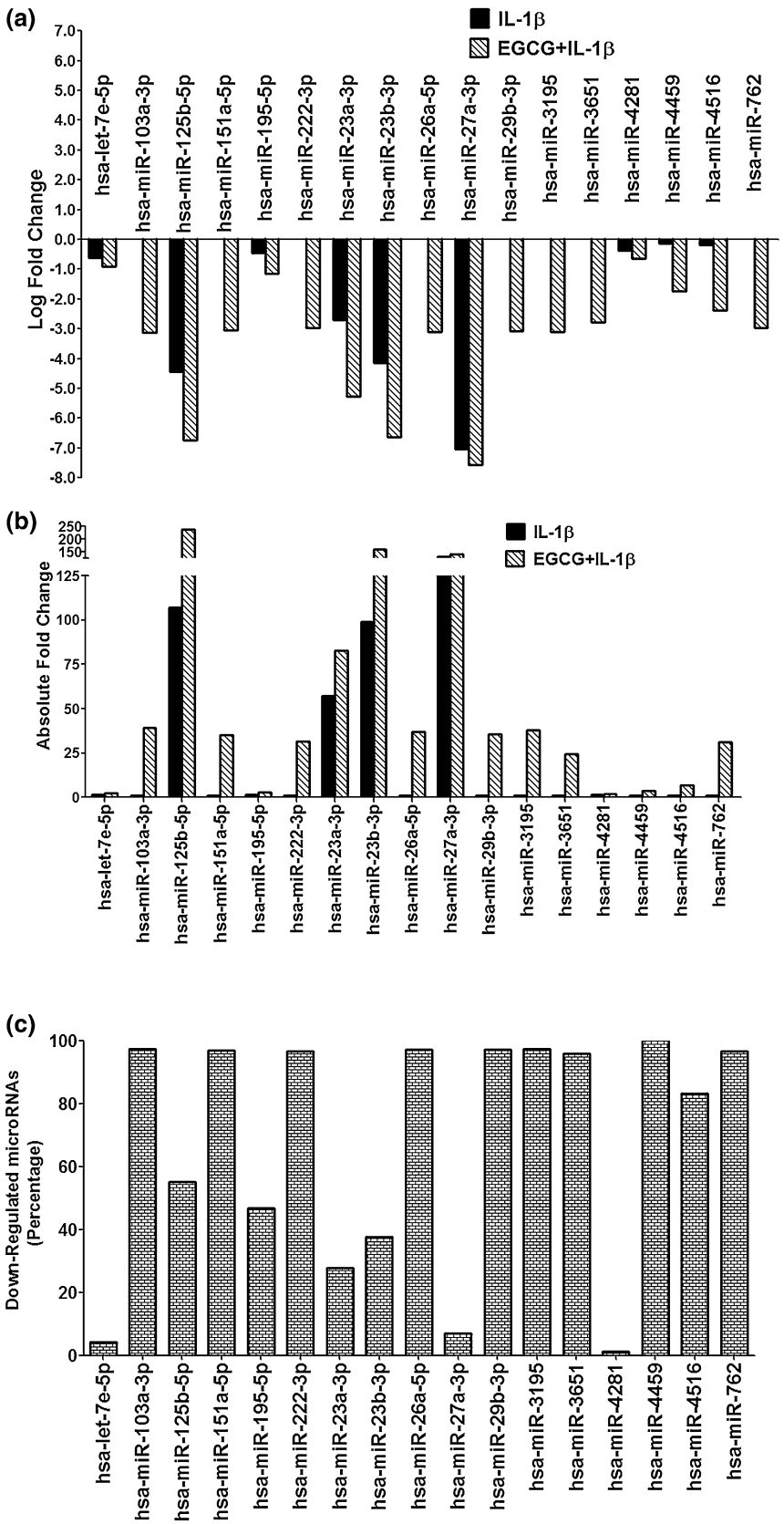
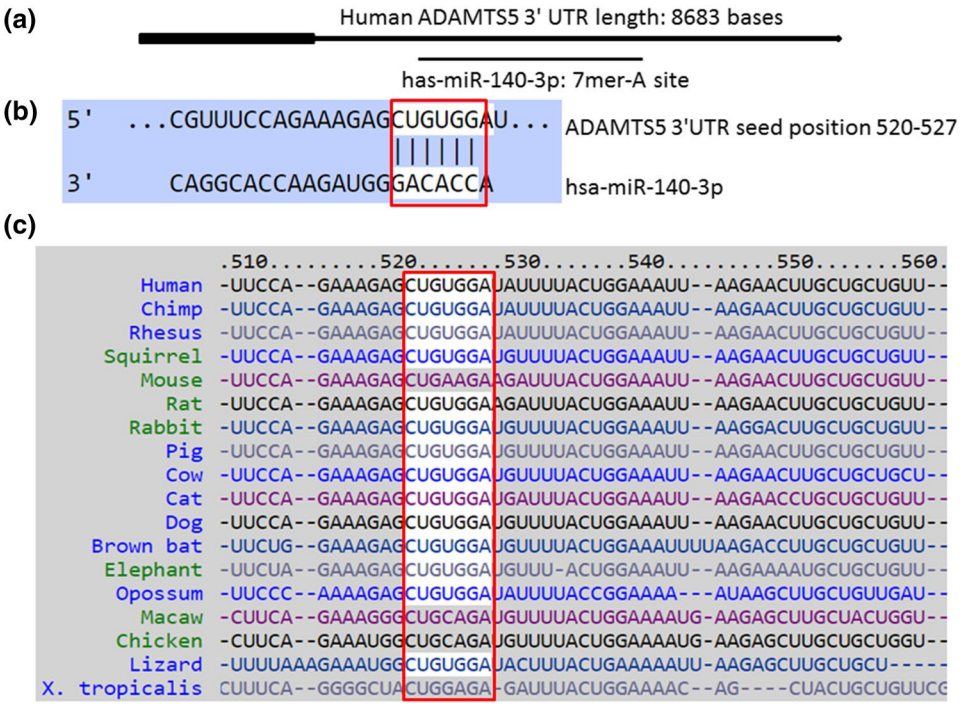


Fig. 3 EGCG down-regulated miRNAs. EGCG-induced down-regulated miRNAs in log fold change (**a**), absolute fold change (**b**), and percent fold change (**c**) in IL-1β-stimulated human OA chondrocytes



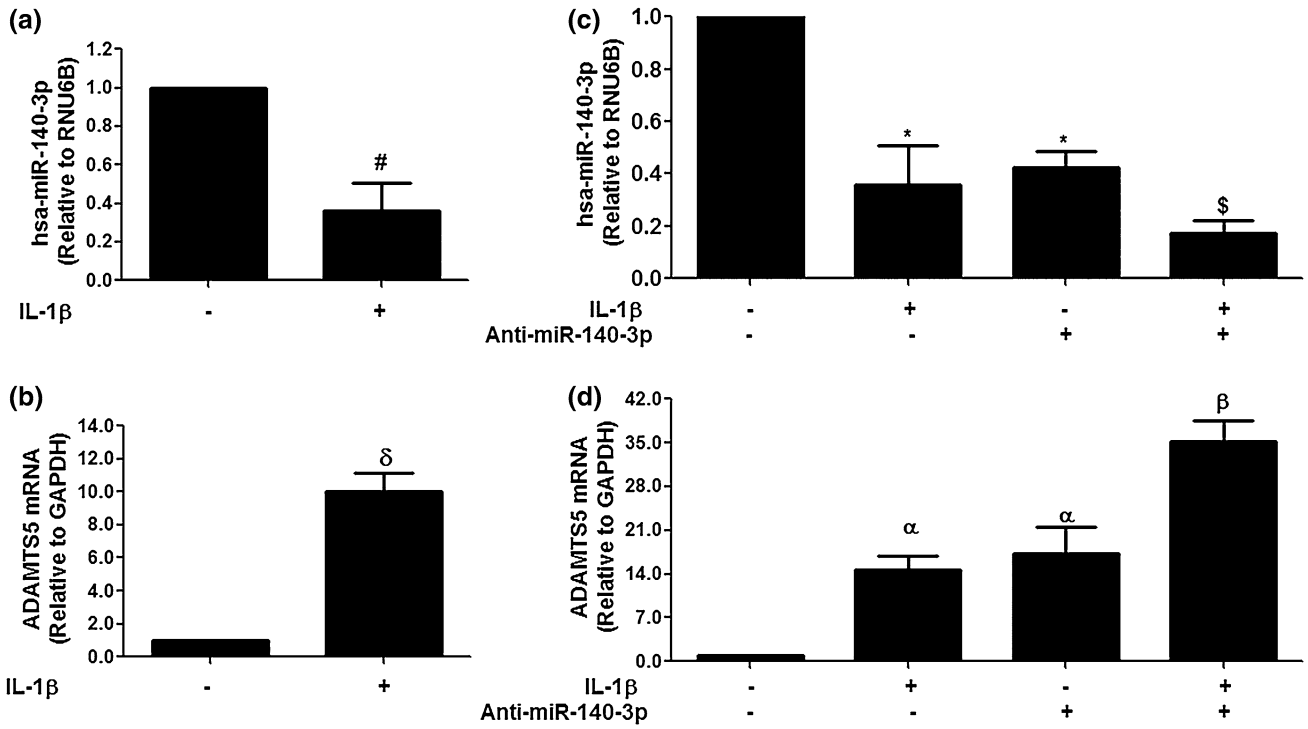


Fig. 4 Seed sequence of hsa-miR-140-3p in 3′UTR of ADAMTS5 mRNA. **a** TargetScan predicted duplex of hsa-miR-140-3p with the seed sequence in the 3′UTR of human ADAMTS5 mRNA. The sequences in *red* are the locations of the potential seed-matched sequence for the miRNAs studied. **b** Seed-matched sequences for hsa-miR-140-3p in the 3′UTR seed position 520–527 of ADAMTS5 mRNA. **c** Cross-species conservation of the hsa-miR-140-3p seed sequence in the 3′UTR of ADAMTS5 mRNA identified by TargetScan algorithm

Fig. 5 IL-1β inhibited hsa-miR-140-3p expression and up-regulated ADAMTS5 mRNA. **a** Expression of miR-140-3p in IL-1β-stimulated human OA chondrocytes determined by TaqMan assays. $^{\#}p < 0.05$ vs. control. **b** ADAMTS5 mRNA expression was determined by TaqMan assay. $^{\delta}p < 0.001$ vs. control. **c** IL-1β-reduced hsa-miR-140-3p expression in anti-miR-140-3p-transfected OA chondrocytes. $^{*}p < 0.01$ vs. control; $^{\$}p < 0.05$ vs. *. **d** IL-1β-induced ADAMTS5 expression in anti-miR-140-3p-transfected OA chondrocytes. $^{\alpha}p < 0.01$ vs. control; $^{\beta}p < 0.05$ vs. α. Unstimulated chondrocytes were used as controls and expression of RNU6B/GAPDH was used as an endogenous control

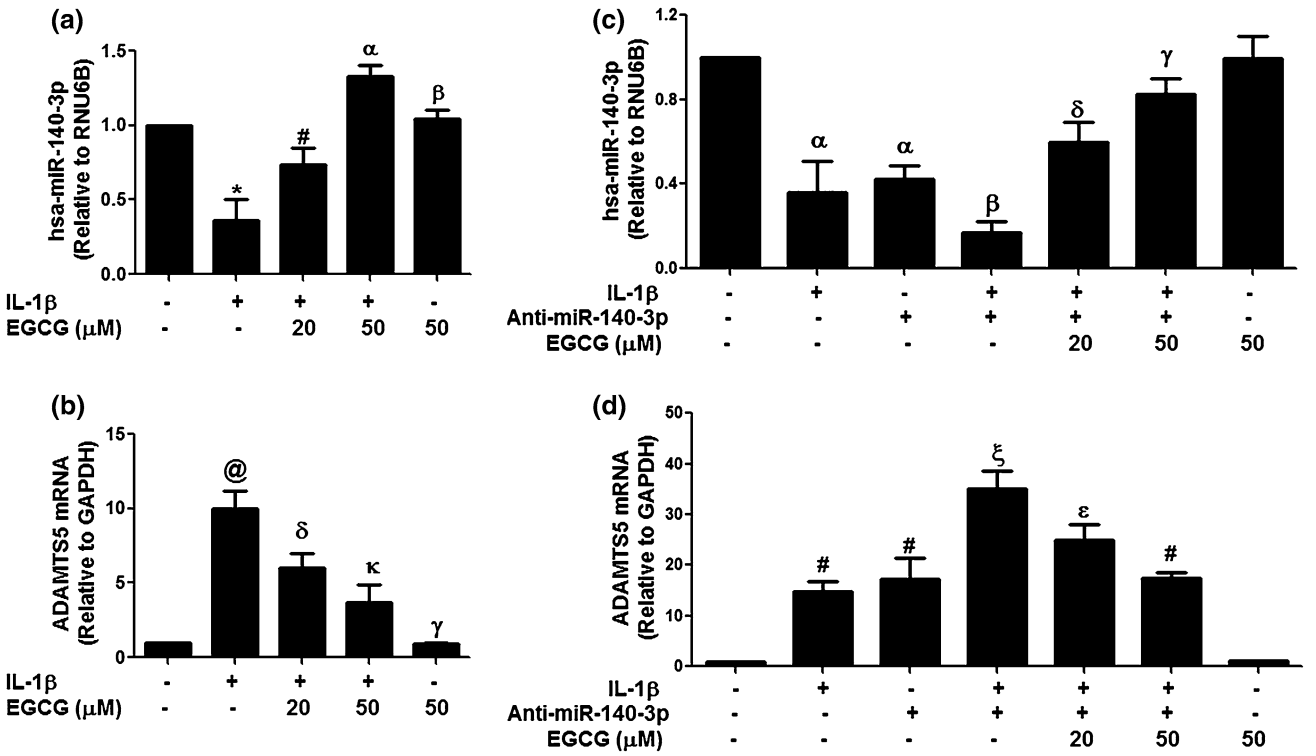


Fig. 6 EGCG up-regulated hsa-miR-140-3p expression and down-regulated ADAMTS5 mRNA. **a** Effect of EGCG on IL-1β-induced down-regulation of hsa-miR-140-3p in human OA chondrocytes determined by TaqMan assays. $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. *; $^{\alpha}p<0.01$ vs. $^{\#}$; $^{\beta}p>0.05$ vs. control. **b** Effect of EGCG on IL-1β-induced up-regulation of ADAMTS5 mRNA expression determined by TaqMan assay. $^{@}p<0.001$ vs. control; $^{@}p<0.05$ vs. δ; $^{\delta}p<0.05$ vs. κ; $^{\gamma}p>0.05$ vs. control. **c** EGCG up-regulates the IL-1β-decreased hsa-miR-140-3p expression in anti-miR-140-3p-transfected OA chondrocytes. Effect of EGCG on IL-1β-decreased hsa-miR-140-3p expression in human OA chondrocytes transfected with anti-miR-140-3p. $^{\alpha}p<0.01$ vs. control; $^{\beta}p<0.05$ vs. α; $^{\delta}p<0.05$ vs. β; $^{\beta}p<0.05$ vs. γ. **d** EGCG down-regulates the IL-1β-induced ADAMTS5 expression in anti-miR-140-3p-transfected OA chondrocytes. Effect of EGCG on IL-1β-induced ADAMTS5 mRNA expression in human OA chondrocytes transfected with anti-miR-140-3p. $^{\#}p<0.01$ vs. control; $^{\xi}p<0.001$ vs. control; $^{\varepsilon}p<0.05$ vs. ξ; $^{\#}p<0.05$ vs. ε. Unstimulated chondrocytes were used as controls and expression of RNU6B/GAPDH was used as an endogenous control

Importantly, transfection of OA chondrocytes with anti-miR-140-3p showed marked increased of IL-1β-induced ADAMTS5 mRNA expression ($p<0.05$) and EGCG significantly inhibited IL-1β-induced ADAMTS5 mRNA in a dose-dependent manner in anti-miR-140-3p-transfected chondrocytes (Fig. 6d, $p<0.05$). In short, these results suggested that EGCG inhibited ADAMTS5 expression via up-regulation of hsa-miR-140-3p expression. These are novel findings and have not been investigated previously.

Discussion

This is the first report to elucidate the effect of EGCG on the global microRNA expression in stimulated human OA chondrocytes. In recent years, there has been concluded that miRNAs play a crucial role in almost all human diseases and now has been suggested that miRNAs considered as potential future therapeutic target for management of human disorders [6, 7]. The role of miRNAs in cartilage homeostasis and in pathogenesis of OA has been well documented [8, 9]. Strong evidences for a key role of IL-1β in OA pathogenesis and altered expression of miRNAs regulating the expression of OA-relevant genes have been reported [8–10]. Miyaki et al. showed that IL-1β inhibited miRNA-140 expression and altered ADAMTS5 expression in OA chondrocytes [40]. Furthermore, they also reported that mice lacking miRNA-140 developed age-related OA-like appearance, characterized by proteoglycan loss and fibrillation of articular cartilage [17]. Recently, we and the others identified many novel miRNAs involved in OA pathogenesis [12–14, 18–20]. These studies indicated strong role of miRNAs in cartilage homeostasis and in the pathogenesis of OA.

Green tea bioactive polyphenol EGCG possessed anti-arthritic activity [21, 22]. Recently, Haqqi and colleagues have shown a wide range of biological effects of EGCG that provide evidence of EGCG-induced modulation in

cartilage protection and inflammation suppressing events in IL-1β-stimulated OA chondrocytes [23]. Earlier, we have also shown that EGCG inhibited the expression of pro-inflammatory mediator's in stimulated human OA chondrocytes [27]. Moreover, several recent reports from different investigators revealed that EGCG has potential to modulate miRNAs expression in various cell types of different diseased patients [28–32]. Recently, we also showed that EGCG inhibited COX-2 expression/PGE_2 production via up-regulation of hsa-miR-199a-3p expression in human OA chondrocytes [33]. In the present study, a spectrum of miRNAs modulatory effects of EGCG has been described for the first time in human chondrocytes. Out of 1347 miRNAs immobilized on the microarray chip, we found 36 differentially expressed miRNAs in chondrocytes treated with EGCG and IL-1β (Tables 1, 2). Out of which EGCG up-regulated expression of 19 miRNAs and down-regulated the expression of 17 miRNAs in stimulated OA chondrocytes. Microarray data were verified by real-time PCR by randomly selected taqman assay for hsa-miRNA-140-3p. Treatment of OA chondrocytes with IL-1β alone significantly decreased expression of has-miR-140-3p; however, EGCG treatment significantly increased hsa-miRNA-140-3p expression. These results indicated the potential of EGCG against IL-1β in regulation of miRNA expression.

To validate our central hypothesis that altered expression of miRNAs may be involved in regulation of disease relevant genes, bioinformatics analysis was performed to determine OA-relevant genes targeted by hsa-miRNA-140-3p. Bioinformatics analysis showed that hsa-miRNA-140-3p seed sequence is complementary to the 3`UTR of ADAMTS mRNA. These findings may indicate that hsa-miRNA-140-3p might be the target of ADAMTS5 by direct recognition of its seed-matched sequence present in its 3`UTR. These bioinformatics predictions were experimentally validated by transfection of OA chondrocytes with anti-hsa-miRNA-140-3p. Our data showed that human OA chondrocytes transfected with anti-miR-140-3p and then treated with IL-1β significantly inhibited miR-140-3p expression as compared with unstimulated transfected chondrocytes. Transfection of OA chondrocytes with anti-miR-140-3p showed marked increased of IL-1β-induced ADAMTS-5 mRNA expression as compared with unstimulated OA chondrocytes, suggested that hsa-miR-140-3p is a direct regulator of ADAMTS5 expression in human OA chondrocytes. Not only these, we also determined that pre-treated of human OA chondrocytes with EGCG significantly enhanced IL-1β-induced hsa-miR-140-3p expression in a dose-dependent manner. However, EGCG alone had no significant effect on hsa-miR-140-3p expression. In these experimental conditions, ADAMTS5 mRNA expression was also quantified and found higher in IL-1β treated OA chondrocytes as compared with control chondrocytes. However, treatment of OA chondrocytes with EGCG showed marked declined of IL-1β-induced ADAMTS5 mRNA expression in a dose-dependent manner. This EGCG-induced negative co-regulation between hsa-miR-140-3p and ADAMTS5 was verified again by transfection of chondrocytes with anti-miR-140-3p. Transfection of OA chondrocytes with anti-miR-140-3p synergized with IL1-β treatment, whereas EGCG treatment consistently enhanced the IL-1β-inhibited hsa-miR-140-3p expression in a dose-dependent manner. Moreover, transfection of OA chondrocytes with anti-miR-140-3p showed marked increased of IL-1β-induced ADAMTS5 mRNA expression. Importantly, treatment of transfected OA chondrocytes with EGCG significantly decreased IL-1β-induced ADAMTS5 mRNA in a dose-dependent manner. Taken together, these data not only suggested that hsa-miRNA-140-3p is a direct regulator of ADAMTS5 expression, but also indicated that EGCG inhibits ADAMTS5 expression via up-regulation of hsa-miRNA-140-3p expression in human OA chondrocytes.

Conclusions

This is the first study to determine the role of bioactive green tea polyphenol EGCG on global miRNAs modulation in stimulated human OA chondrocytes. The study suggested the potential of EGCG in OA treatment/prevention that may be related to its ability to globally modulate microRNA expression. Moreover, the present study also concluded that EGCG inhibited IL-1β-induced ADAMTS5 expression via up-regulation of the expression of hsa-miR-140-3p in human OA chondrocytes. These findings support the use of EGCG as an anti-arthritic agent for prevention/treatment of OA or other degenerative disorders.

Acknowledgements This study was supported by the National Science, Technology and Innovation Plan (NSTIP) Grant #11-BIO1885-09 from Qassim University, KSA. Support from Professor Hani A. Al-Shobaili (Dean, College of Medicine, Qassim University, KSA) is acknowledged and greatly appreciated.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Troeberg L, Nagase H (2012) Proteases involved in cartilage matrix degradation in osteoarthritis. Biochim Biophys Acta 1824:133–145
2. Apte SS (2016) Anti-ADAMTS5 monoclonal antibodies: implications for aggrecanase inhibition in osteoarthritis. Biochem J 473:e1–e4
3. Huang K, Wu LD (2008) Aggrecanase and aggrecan degradation in osteoarthritis: a review. J Int Med Res 36:1149–1160

4. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, Morris EA (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 434:644–648
5. Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma HL, Flannery CR, Kanki K, Wang E, Peluso D, Yang Z, Majumdar MK, Morris EA (2004) Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. Arthritis Rheum 50:2547–2558
6. Pasquinelli AE (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13:271–282
7. Lovendorf MB, Skov L (2015) Epigenetics and dermatology. Chapter 9—MicroRNAs in skin diseases. pp 177–205
8. Sondag GR, Haqqi TM (2016) The role of microRNAs and their targets in osteoarthritis. Curr Rheumatol Rep 18:56
9. Mirzamohammadi F, Papaioannou G, Kobayashi T (2014) MicroRNAs in cartilage development, homeostasis, and disease. Curr Osteoporos Rep 12:410–419
10. Kobayashi T, Lu J, Cobb BS, Rodda SJ, McMahon AP, Schipani E, Merkenschlager M, Kronenberg HM (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. Proc Natl Acad Sci USA 105:1949–1954
11. Miyaki S, Sato T, Inoue A, Otsuki S, Ito Y, Yokoyama S, Kato Y, Takemoto F, Nakasa T, Yamashita S, Takada S, Lotz MK, Ueno-Kudo H, Asahara H (2010) MicroRNA-140 plays dual roles in both cartilage development and homeostasis. Genes Dev 24:1173–1185
12. Rasheed Z, Al-Shobaili HA, Rasheed N, Al Salloom AA, Al-Shaya O, Mahmood A, Alajez NM, Alghamdi AS, Mehana EE (2016) Integrated study of globally expressed microRNAs in IL-1β-stimulated human osteoarthritis chondrocytes and osteoarthritis relevant genes: a microarray and bioinformatics analysis. Nucleosides Nucleotides Nucleic Acids 35:335–355
13. Akhtar N, Rasheed Z, Ramamurthy S, Anbazhagan AN, Voss FR, Haqqi TM (2010) MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes. Arthritis Rheum 62:1361–1371
14. Rasheed Z, Al-Shobaili HA, Rasheed N, Mahmood A, Khan MI (2016) MicroRNA-26a-5p regulates the expression of inducible nitric oxide synthase via activation of NF-κB pathway in human osteoarthritis chondrocytes. Arch Biochem Biophys 594:61–67
15. Meng F, Zhang Z, Chen W, Huang G, He A, Hou C, Long Y, Yang Z, Zhang Z, Liao W (2016) MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1β-induced chondrocyte responses. Osteoarthritis Cartilage 24:932–941
16. Hou C, Meng F, Zhang Z, Kang Y, Chen W, Huang G, Fu M, Sheng P, Zhang Z, Liao W (2015) The role of MICRORNA-381 in chondrogenesis and interleukin-1-β induced chondrocyte responses. Cell Physiol Biochem 36:1753–1766
17. Makki MS, Haseeb A, Haqqi TM (2015) MicroRNA-9 promotion of interleukin-6 expression by inhibiting monocyte chemoattractant protein-induced protein 1 expression in interleukin-1β-stimulated human chondrocytes. Arthritis Rheumatol 67:2117–2128.
18. Akhtar N, Makki MS, Haqqi TM (2015) MicroRNA-602 and microRNA-608 regulate sonic hedgehog expression via target sites in the coding region in human chondrocytes. Arthritis Rheumatol 67:423–434.
19. Park SJ, Cheon EJ, Lee MH, Kim HA (2013) MicroRNA-127-5p regulates matrix metalloproteinase 13 expression and interleukin-1β-induced catabolic effects in human chondrocytes. Arthritis Rheum 65:3141–3152
20. Park SJ, Cheon EJ, Kim HA (2013) MicroRNA-558 regulates the expression of cyclooxygenase-2 and IL-1β-induced catabolic effects in human articular chondrocytes. Osteoarthritis Cartilage 21:981–989
21. Rasheed Z (2016) Green tea bioactive polyphenol epigallocatechin-3-o-gallate in osteoarthritis: current status and future perspectives. Int J Health Sci (Qassim) 10:V-VIII.
22. Haqqi TM, Anthony DD, Gupta S, Ahmad N, Lee MS, Kumar GK, Mukhtar H (1999) Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. Proc Natl Acad Sci USA 96:4524–4529
23. Akhtar N, Haqqi TM (2011) Epigallocatechin-3-gallate suppresses the global interleukin-1beta-induced inflammatory response in human chondrocytes. Arthritis Res Ther 13:R93
24. Singh R, Akhtar N, Haqqi TM (2010) Green tea polyphenol epigallocatechin-3-gallate: inflammation and arthritis. Life Sci 86:907–918
25. Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM (2004) Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1 beta-induced expression of matrix metalloproteinase-1 and—13 in human chondrocytes. J Pharmacol Exp Ther 308:767–773
26. Ahmed S, Rahman A, Hasnain A, Lalonde M, Goldberg VM, Haqqi TM (2002) Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-1 beta-induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes. Free Radic Biol Med 33:1097–1105
27. Rasheed Z, Anbazhagan AN, Akhtar N, Ramamurthy S, Voss FR, Haqqi TM (2009) Green tea polyphenol epigallocatechin-3-gallate inhibits advanced glycation end product-induced expression of tumor necrosis factor-alpha and matrix metalloproteinase-13 in human chondrocytes. Arthritis Res Ther 11:R71
28. Zhu K, Wang W (2016) Green tea polyphenol EGCG suppresses osteosarcoma cell growth through upregulating miR-1. Tumour Biol 37:4373–4382
29. Sethi S, Li Y, Sarkar FH (2013) Regulating miRNA by natural agents as a new strategy for cancer treatment. Curr Drug Targets 14:1167–1174
30. Tsang WP, Kwok TT (2010) Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells. J Nutr Biochem 21:140–146
31. Jiang L, Tao C, He A, He X (2014) Overexpression of miR-126 sensitizes osteosarcoma cells to apoptosis induced by epigallocatechin-3-gallate. World J Surg Oncol 12:383
32. Yamada S, Tsukamoto S, Huang Y, Makio A, Kumazoe M, Yamashita S, Tachibana H (2016) Epigallocatechin-3-O-gallate up-regulates microRNA-let-7b expression by activating 67-kDa laminin receptor signaling in melanoma cells. Sci Rep 6:19225.
33. Rasheed Z, Rasheed N, Al-Shobaili HA (2016) Epigallocatechin-3-O-gallate up-regulates microRNA-199a-3p expression by down-regulating the expression of cyclooxygenase-2 in stimulated human osteoarthritis chondrocytes. J Cell Mol Med 20:2241–2248
34. Rasheed Z, Haqqi TM (2012) Endoplasmic reticulum stress induces the expression of COX-2 through activation of eIF2α, p38-MAPK and NF-κB in advanced glycation end products stimulated human chondrocytes. Biochim Biophys Acta 1823:2179–2189
35. Rasheed Z, Akhtar N, Haqqi TM (2011) Advanced glycation end products induce the expression of interleukin-6 and interleukin-8 by receptor for advanced glycation end product-mediated activation of mitogen-activated protein kinases and nuclear factor-κB in human osteoarthritis chondrocytes. Rheumatology (Oxford) 50:838–851
36. Rasheed Z, Akhtar N, Haqqi TM (2010) Pomegranate extract inhibits the interleukin-1β-induced activation of MKK-3,

p38α-MAPK and transcription factor RUNX-2 in human osteoarthritis chondrocytes. Arthritis Res Ther 12:R195
37. Agarwal V, Bell GW, Nam JW, Bartel DP (2015) Predicting effective microRNA target sites in mammalian mRNAs. Elife Aug 12;4.
38. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
39. Qureshi HY, Ahmad R, Zafarullah M (2008) High-efficiency transfection of nucleic acids by the modified calcium phosphate precipitation method in chondrocytes. Anal Biochem 382:138–140
40. Miyaki S, Nakasa T, Otsuki S, Grogan SP, Higashiyama R, Inoue A, Kato Y, Sato T, Lotz MK, Asahara H (2009) MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. Arthritis Rheum 60:2723–2730