Osteoarthritis and Cartilage (2009) **17,** 464–472
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doi:10.1016/j.joca.2008.09.012

Osteoarthritis and Cartilage

ICRS International Cartilage Repair Society

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The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-α and MMP13

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Summary

Objective: To identify differentially expressed microRNAs (miRNAs) in human osteoarthritic (OA) cartilage and bone tissue and to determine their relevance to chondrocyte function.

Methods: Cartilage and bone was obtained from OA patients who underwent total knee joint replacement surgery or from post-mortem patients with no previous history of OA. MiRNA expression was quantified by real-time PCR (RT-PCR). Functional pathway analysis of miRNA was performed using Ingenuity Pathway® Analysis. Primary chondrocytes were isolated by collagenase digestion and transfected with miRNA mimics and miRNA inhibitors using cationic lipid. Tumour Necrosis Factor-alpha (TNF-α) and Matrix metalloprotease 13 (MMP13) protein levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA).

Results: In total we identified 17 miRNA that showed greater than 4-fold differential expression between OA and normal cartilage, and 30 miRNA that showed greater than 4-fold differential expression in OA bone. Functional pathway analysis of the predicted gene targets for miR-9, miR-98, which were upregulated in both OA bone and cartilage tissue, and miR-146, which was downregulated in OA cartilage, suggested that these miRNA mediate inflammatory functions and pathways. Over-expression of miR-9, miR-98 or miR-146 in isolated human chondrocytes reduced interleukin-1beta (IL-1β) induced TNF-α production. Furthermore, inhibition and over-expression of miR-9 modulated MMP13 secretion.

Conclusions: We have identified a number of differentially expressed miRNAs in late-stage human OA cartilage and bone. Functional analysis of miR-9, miR-98 and miR-146 in primary chondrocytes suggests a role in mediating the IL-1β induced production of TNF-α. MiR-9, upregulated in OA tissue, was found to inhibit secretion of the collagen type II-targeting metalloproteinase MMP13 in isolated human chondrocytes.
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Key words: microRNA, miRNA, Osteoarthritis, Cartilage, Chondrocytes.

Introduction

MicroRNAs (miRNAs) are small non-coding RNA species that bind to target mRNAs and prevent their translation into proteins through a process entitled RNA interference[1]. Predictive algorithms suggest that up to a third of all human genes contain putative single or multiple miRNA recognition elements[2]. Importantly, the finding that miRNAs might regulate multiple genes and the demonstration of differential expression in animal cells and tissues[3,4] has led to speculation that their control of gene expression is conceptually similar to the action of transcription factors[5] although they act at the translational level.

Greater than 700 miRNAs have so far been identified and initial functional studies have demonstrated roles for specific miRNA across a range of biological processes including apoptosis/proliferation[6,7], development[8–10] and glucose and lipid metabolism[11], whilst differential expression of miRNAs have been reported in multiple cancers[12,13]. Recent work by Kobayshi and colleagues has demonstrated the importance of miRNA in cartilage function[14]. They generated cartilage-specific Dicer-null mice, Dicer is a critical enzyme in the miRNA biogenesis pathway. The growth plates of these mice showed greatly decreased chondrocyte proliferation and accelerated hypertrophy, leading to severe growth defects. Hence miRNAs are essential for normal chondrocyte development and function in the mice. However, at present, little is known about either the expression or the functional role of miRNA in osteoarthritis (OA). Recently, it was reported that the cartilage-specific miRNA140 targets histone deacetylase 4 mRNA[15] and the authors proposed a role for miR140 in long bone development. Since inflammation is a feature of OA, with evidence of synovitis in early OA[16,17] and the presence of elevated levels of pro-inflammatory cytokines in synovial fluid, the recent reports that some miRNA mediate inflammatory pathways are of great interest. Indeed, it was recently demonstrated that miRNA are rapidly and transiently upregulated in response to LPS-induced inflammation *in vitro*[18,19] and *in vivo* in the mouse lung[20], whilst expression profiling of miRNA in the inflammatory disorders psoriasis and atoptic eczema identified specific

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Received 13 May 2008; revision accepted 25 September 2008.

miRNA which were differentially expressed in the inflammed condition[21,22]. Furthermore, a potential role of miRNAs in the adaptive immune response has been demonstrated from studies using knockout mice. These studies showed reduced CD3/CD28-induced IFNγ release from CD4 + T-cells[18] and B cell receptor-mediated TNFα production from B-cells[23], indicating that miR-155 facilitates or positively regulates cytokine release in lymphoid cells. Similarly, increased miR-181a expression was also shown to augment IL-2 release following activation of T-cells[24]. Interestingly, recent reports have also shown increased miR-155, miR-146a and miR-146b expression following activation of the innate immune response in monocytes/macrophages[18,19].

In this study we have, for the first time, profiled the expression of miRNA in human OA and normal cartilage and bone. In addition, we have examined the physiological role of specific miRNA using both a bioinformatics approach and through the use of miRNA mimics and inhibitors to modulate both miRNA expression and activity in primary human chondrocytes to determine their effect on specific catabolic pathways.

Materials and methods

PREPARATION OF HUMAN CARTILAGE AND BONE FROM DONOR KNEES

Human OA cartilage and bone was obtained following total knee replacement operations (age range of between 66 and 71 years), whilst normal cartilage and bone was taken from post-mortem donors (age range of between 63 and 70 years) with no previous history of joint pain ($n = 3$ cartilage; $n = 4$ bone). Full ethical consent was obtained from all donors and families.

ISOLATION OF PRIMARY HUMAN CHONDROCYTES

To isolate primary chondrocytes, slices of cartilage were stripped from the femoral condyles and tibial plateaux, cut into small pieces using a scalpel blade and digested in growth media (DMEM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, non-essential amino acids (Invitrogen) and amphotericin (2.5 μg/ml)) containing 1% collagenase type IA (0.5−3.0 FALGPA units/mg, Sigma, Dorset, UK). Following overnight incubation at 37°C, the sample was filtered to remove undigested cartilage and chondrocyte cells pelleted at 2000 g for 5 min before being resuspended in growth media and grown in monolayer.

MiRNA ISOLATION AND QUANTIFICATION

Cartilage and bone tissue (0.5 g) from the knees of OA and normal donors was crushed to a powder using a Freezer Mill (Glen Creston, London, UK.) and total RNA (including the microRNA fraction) extracted by homogenisation using a polytron in 6 vol of Trizol reagent (Invitrogen, UK). Total RNA was also extracted from primary human chondrocytes by adding Trizol reagent directly to the chondrocyte cells grown on tissue culture plates. RNA was resuspended in 50 μl RNase-free water (Promega UK, Southampton, UK) and quantified by using a spectrophotometer (Nanodrop) and using an Agilent Bioanalyser to confirm quality. MiRNA expression profiling was carried out on total RNA extracts by two-step Taqman® RT-PCR, normalised to 18S. Reverse transcription was carried out on 5 ng of total RNA in 7.5 μl reactions using the TaqMan® MicroRNA Reverse Transcription Kit using RT stem-loop primers (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The expression profile of 157 human miRNA was then determined using the Human Panel Early Access kit (Applied Biosystems) according to the manufacturer's instructions. The reaction was carried out using a 7700 Real Time PCR system (Applied Biosystems) in 10 μl reaction volumes. In a separate reaction, 1-step PCR (Qiagen) was performed on the total RNA for 18S quantification, which was used as the endogenous control for data normalisation.

FUNCTIONAL PATHWAY ANALYSIS OF SPECIFIC miRNAs

Computationally predicted gene targets for miR-9, miR-98 and miR-146 were obtained from the MicroCosm web resource (Sanger) using the miRanda algorithm. Selecting the top 200 target sequences based on the confidence of the hit we uploaded these gene targets into the Ingenuity Pathway Analysis application (Ingenuity® Systems, www.ingenuity.com) to generate a network of candidate miRNA gene targets. Functional analysis of the network was then performed to identify the biological functions and/or diseases that were most significant to the genes in the network. Fisher's exact test was used to calculate the probability that each biological function and/or disease assigned to that network was due to chance alone. Canonical pathways analysis was performed to identify pathways from the Ingenuity® Pathway Analysis library of canonical pathways that were most significant to the genes in the network. The significance of the association of a given canonical pathway and the network of gene targets was measured in two ways. Firstly, by the ratio of the number of genes in the network that mapped to the canonical pathway divided by the total number of genes that map to the canonical pathway. Secondly, Fisher's exact test was used to calculate a P-value of the association between the genes in the network and the canonical pathway.

MiRNA MODULATION USING miRNA MIMICS AND INHIBITORS

Human primary OA chondrocytes were transfected with pre-miRNA mimics (Applied Biosystems) or with Locked Nucleic Acid (LNA) miRNA inhibitors (Exiqon) using cationic Atufect lipid (Atugen) for 24 h. Following transfection cells were stimulated with IL-1β for 24 h and the cell supernatants harvested for subsequent protein analysis by ELISA.

ANALYSIS OF THE PRODUCTION OF TNFα AND MMP13 PROTEIN

Conditioned media from transfected cells was assayed for levels of TNF-α using a commercially available ELISA kit (R&D systems). In brief, polystyrene high-binding plates (Costar) were coated overnight at 4°C with capture antibody diluted 1:500 to 4 μg/ml. After removal of the coating antibody, plates were blocked in Phosphate Buffered Saline (PBS) containing 1% BSA before addition of 40 μl of either sample or standard together with the detection antibody 20 μl/well diluted 1:1000. Plates were then left a further 2 h at room temperature before the addition of Streptavidin HRP. MMP13 protein levels were determined using an in-house ELISA utilising a polyclonal MMP13 antibody (Abcam; ab9128), diluted 1:2500 in PBS and a secondary anti-rabbit HRP conjugate (GE Healthcare; NA934). All plates were developed with the addition of 40 μl/well of TMB for 20 min in the dark at room temperature, and reactions stopped with the addition of H_2SO_4 (2M) before the absorbance read on a plate reader (Pherostar).

STATISTICAL ANALYSIS

All data are reported as means ± S.E.M. Comparisons were performed using Analysis of variance (ANOVA) and post-hoc tests used to test for significance, with significance accepted as $P < 0.05$.

Results

MiRNA ARE DIFFERENTIALLY EXPRESSED IN OA BONE AND CARTILAGE

In order to obtain sufficient quantity of miRNA for subsequent quantification analysis it was necessary to extract total RNA from cartilage and bone tissue using Trizol reagent. A comparison of the Bioanalyser gel profile from the total RNA extraction with the gel profile of isolated miRNA using the mirVana kit shows that total RNA extractions using Trizol retain the miRNA fraction (Fig. 1).

Expression profiling of 157 human miRNA extracted from OA and normal cartilage ($n = 3$) identified 17 miRNA which showed differential expression of greater than 4-fold between diseased and normal tissue (Table I). Among the most notable changes were miR-9, miR-25 and miR-98, which were upregulated by 8-, 8- and 23-fold respectively ($P < 0.05$), miR-146, which was downregulated by 14-fold ($P < 0.05$) and miR-149 that was downregulated 27 fold ($P < 0.01$, Table I).

For reference, we also examined the miRNA profile of OA and normal bone. We found that 30 miRNA were identified with differential expression of greater than 4-fold between diseased and normal bone tissue (Table I). Of these the miRNA showing the greatest fold change were miR27a, miR34b and miR-98, which were upregulated in OA bone

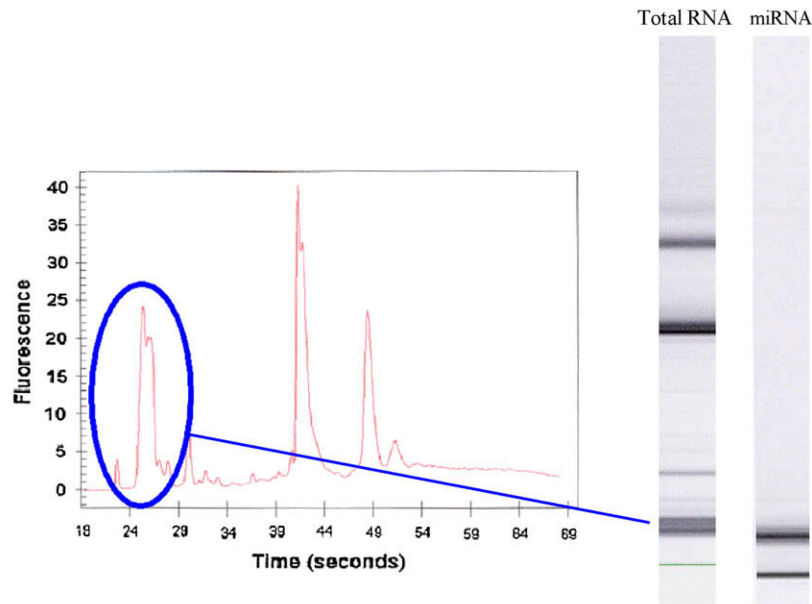

Fig. 1. Isolation of miRNA fraction in human OA tissue. A comparison of BioAnalyser gel profiles for total RNA extraction using Trizol and miRNA extraction when isolated using the *mir*Vana kit. MiRNA fraction can be seen in the total RNA prep.

by 33-, >100- and >100-fold respectively and miR330 which was downregulated by 4-fold in OA bone ($P < 0.05$). Of particular interest was the finding that miR-9 and miR-98, which we had previously identified as being upregulated in OA cartilage, were also upregulated in OA bone (Table I).

DIFFERENTIALLY EXPRESSED miRNA MAY MEDIATE INFLAMMATORY PATHWAYS

In determining the functional role of differentially expressed miRNA in OA we focussed on miR-9, miR-98 and miR-146. MiR-9 and miR-98 were selected as highly upregulated miRNA species and because they were also strongly upregulated in OA bone tissue. MiR-146 was selected because it was highly downregulated in OA cartilage and the evidence from literature that miR-146 may play a role in inflammation[18,19].

In order to explore the functional relevance of the selected miRNA species in human chondrocytes we undertook a bioinformatics analysis approach to guide experimental design. The principle aim of this work was not to identify specific miRNA gene targets and establish mechanistic linkages but rather to determine the functional relevance of miRNA on OA-relevant chondrocyte phenotypes. Firstly, we performed functional pathway analysis of the top 200 (based on confidence hit) predicted mRNA gene targets of miR-9, miR-98 and miR-146 using the Ingenuity® Pathway Analysis application to identify significant biological functions and canonical pathways. Core functional analysis revealed that for both miR-9 and miR-98 (which were upregulated in OA bone and OA cartilage) one of the most significant biological functions was inflammatory disease, with a total of 38 and 32 gene targets of miR-9 and miR-98 respectively linked to inflammation and inflammatory disorders, including arthritis [Figs. 2(a) and 3(a)]. Of these putative gene targets 7 were common to both miR-9 and miR-98 namely alpha-1-microglobulin/bikunin precursor (AMBP), Bux non-receptor tyrosine kinase (BMX), apolipoprotein E (APOE), major histocompatability

Table 1

*MiRNA with ≥4-fold differential expression between OA and normal tissue. Positive fold change values indicate elevated expression in OA tissue, whilst negative fold change values indicate elevated expression in normal tissue. * = significantly different from normal control value*

miRNA	Cartilage		miRNA	Bone	
	Fold Change	*P* Value		Fold Change	*P* Value
miR-9	8	0.04*	miR-9	4	0.03*
miR-25	8	0.03*	miR-19a	4	0.18
miR-34a	4	0.49	miR-21	19	0.06
miR-34b	8	0.29	miR-23a	4	0.17
miR-98	23	0.02*	miR-27a	33	0.08
miR-107	−4	0.01*	miR-27b	5	0.09
miR-130b	−7	0.36	miR-31	21	0.07
miR-137	5	0.54	miR-34b	>100	0.04*
miR-146	−14	0.04*	miR-34c	21	0.1
miR-148	−5	0.28	miR-98	>100	0.02*
miR-149	−27	<0.01*	miR-100	7	0.14
miR-182	4	0.48	miR-104	23	0.04*
miR-185	4	0.12	miR-105	4	0.02*
miR-200a	11	<0.01*	miR-122a	9	0.04*
miR-211	5	<0.01*	miR-135a	4	0.02*
miR-299	5	0.02*	miR-135b	5	0.01*
miR-342	4	0.06	miR-139	6	<0.01*
			miR-142-3	5	0.06
			miR-144	5	0.04*
			miR-147	8	0.01*
			miR-148	20	0.04*
			miR-149	11	0.03*
			miR-181a	4	0.15
			miR-187	8	0.11
			miR-210	5	0.053
			miR-299	5	0.11
			miR-302d	22	0.04*
			miR-330	−4	0.08
			miR-335	12	0.08
			miR-340	9	0.36

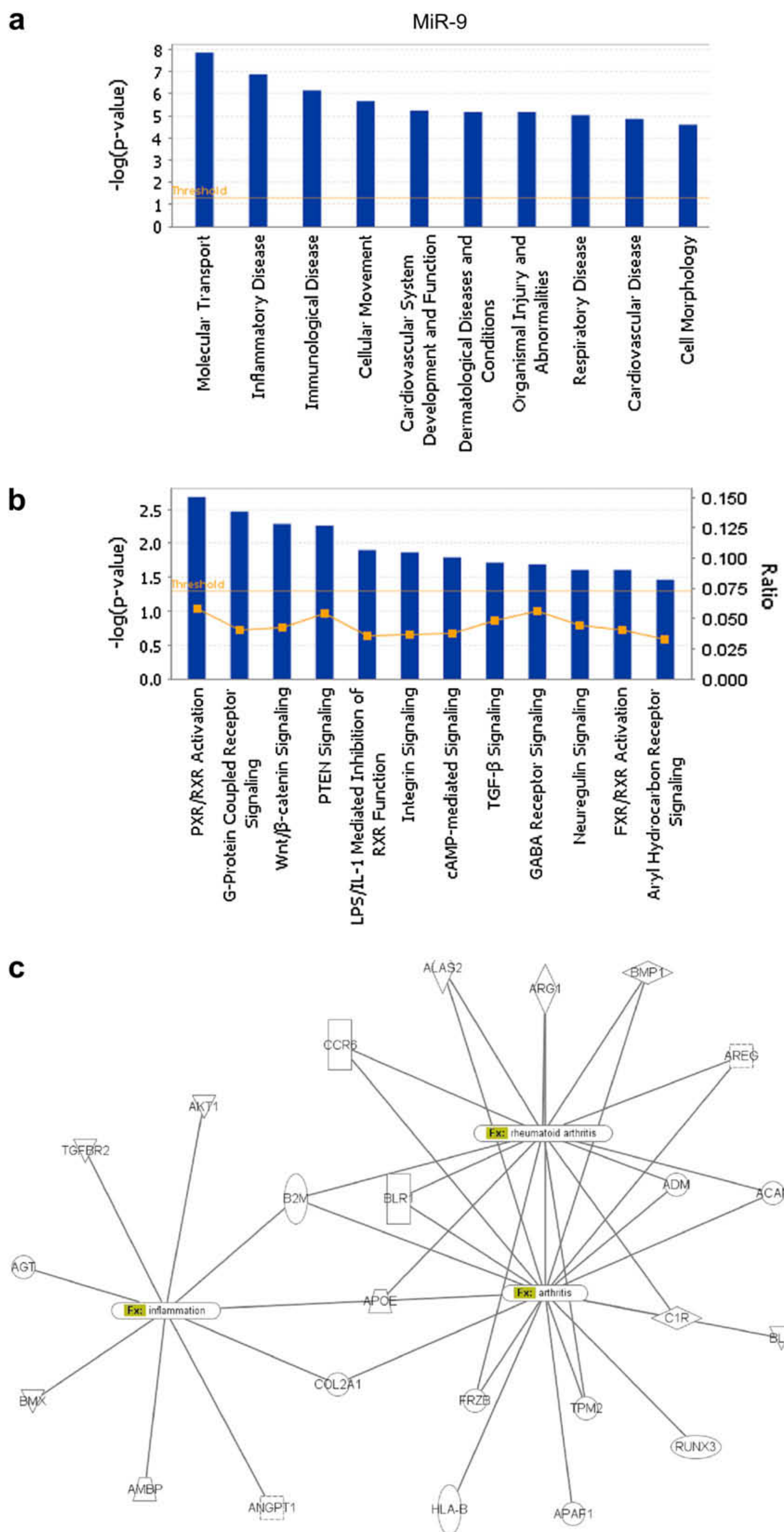

Fig. 2. Pathway analysis of miR-9. Core functional pathway analysis using Ingenuity Pathway Analysis application of the top 200 predicted gene targets of miR-9. (**a**) Most significant biological functions mapped to the network of miR-9 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3. (**b**) Most significant canonical signalling pathways from the Ingenuity canonical pathway library mapped to the network of miR-9 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3. Line shows ratio of genes in network to total number of genes in canonical pathway. (**c**) Network of miR-9 gene targets linked to biological functions inflammation, rheumatoid arthritis and arthritis.

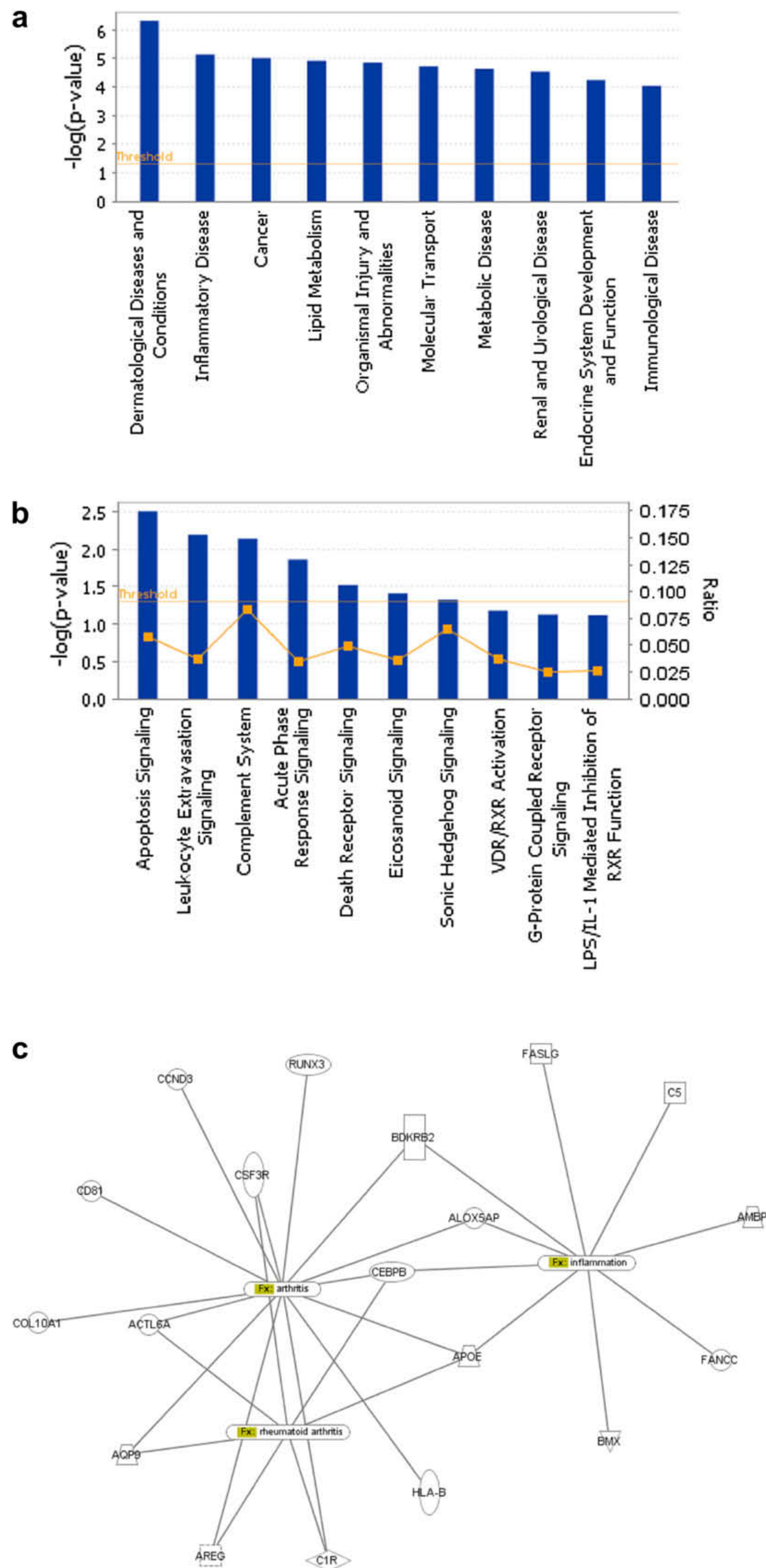

Fig. 3. Pathway analysis of miR-98. Core functional pathway analysis using Ingenuity Pathway Analysis application of the top 200 predicted gene targets of miR-98. (**a**) Most significant biological functions mapped to the network of miR-98 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3. (**b**) Most significant canonical signalling pathways from the Ingenuity canonical pathway library mapped to the network of miR-98 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3. Line shows ratio of genes in network to total number of genes in canonical pathway. (**c**) Network of miR-98 gene targets linked to biological functions inflammation, rheumatoid arthritis and arthritis.

complex Class I, B (HLA-B), Runt-related transcription factor 3 (RUNX3), complement component 1, subcomponent (C1R) and amphiregulin (AREG) [Figs. 2(c) and 3(c)].

Canonical pathway analysis was then used to identify pathways from the Ingenuity® Pathway Analysis library that were most significant to the predicted miRNA gene targets. Canonical pathway analysis of the miR-9 gene targets found that the three most significant pathways were PXR/RXR activation, G-protein coupled receptor (GPCR) signalling and Wnt/β-catenin signalling, whilst for miR-98 the most significant pathways were apoptosis signalling, leukocyte extravasation signalling and complement system. Strikingly, canonical pathway analysis of the top 200 predicted gene targets of miR-146 mapped only one pathway that was clearly significant, which was the NFκB signalling pathway with a total of 9 genes potentially targeted by miR-146 present in the pathway [Fig. 4(b), (c)]. As stated above, this bioinformatics approach utilises predicted genes targets based on the miRANDA alogithim and therefore a degree of caution should be taken in interpreting these findings. However, the mapping of miR-9, miR-98 and miR-146 gene targets to inflammatory pathways (including the NFkB signalling pathway) suggested that determining the effect of modulating these microRNA on TNF-α and MMP13 production would be of significant interest given the these functionally OA relevant end-points are known to be mediated by inflammatory pathways[25].

DIFFERENTIALLY EXPRESSED miRNA MEDIATE THE SECRETION OF TNF-α AND MMP13 PROTEIN IN HUMAN PRIMARY CHONDROCYTES

In order to further study the functional role of miR-9, miR-98 and miR-146 we modulated both the expression and activity of these miRNA in chondrocytes and examined the effect on two functional biological assays representative of inflammation pathways: TNF-α and a gene target regulated by TNF-α of particular relevance to OA disease, namely matrix metalloproteinase-13 (MMP13). IL-1β is an activator of NFκB and a cytokine associated with OA; thus, the effects of miRNA modulation on TNF-α and MMP-13 protein production were explored both at a basal level and following induction with IL-1β.

Using LNA inhibitors of miR-9, miR-98 and miR-146, basal TNF-α protein was marginally elevated by between 21 and 48% at the highest concentration relative to a control LNA miRNA inhibitor (Fig. 5a). Consistent with this, over-expression of the each of the miRNA species, had no effect on basal TNF-α levels. However, over-expression of miRNAs significantly attenuated IL-1β inducted TNF-α by 40%, 62% and 56% ($P < 0.05$) for miR-9, miR-98 and miR-146 respectively [Fig. 5(b)].

We next examined the effect of miRNA modulation on the secretion of MMP13 protein by chondrocytes and found that upon inhibition of miR-9 by specific LNA there was a trend to an elevation of basal MMP13 protein levels and a significant increase ($P < 0.05$) in the IL-1β induction of MMP13 protein [Fig. 6(a)]. Conversely, when miR-9 was over-expressed both basal and IL-1β-induced MMP13 levels were reduced [Fig. 6(b)]. Inhibition and over-expression of miR-98 and miR-146 did not appear to have any effect on either basal or IL-1β-induced MMP13 levels [Fig. 6(a) and (b)] respectively.

Discussion

In attempting to understand the biological pathways and processes that underlie the pathogenesis and progression of osteoarthritic disease genomic approaches have identified genes associated with the extraceullular matrix, oxidative stress, phenotype stabilisation and IL-1 signalling as being differentially expressed in OA disease[26–28]. Our understanding of how these key pathways are regulated and how they become dysregulated in disease is critical in attempting to develop novel therapeutic entities. In this context, the identification of endogenously expressed miRNA species that regulate the post-transcriptional expression of multiple genes and possibly therefore whole biological pathways and processes is of great interest.

In this study we have profiled the expression of 157 human miRNA and identified a number of miRNAs that are differentially expressed in human OA cartilage and OA bone compared to non-diseased tissue. Focussing on miR-9 and miR-98 (which were upregulated in both OA cartilage and OA bone tissue), and miR-146 (which was downregulated in OA cartilage) we have performed a bioinformatics approach, which indicated that miR-9 and miR-98 have the potential to target a significant number of genes involved in inflammatory diseases, whilst miR-146 was associated with a significant number of genes within the NFκB pathway.

We found that miR-9 was upregulated in OA cartilage, and furthermore that over-expression in isolated chondrocytes decreased MMP13 secretion, while inhibition of miR-9 increased levels of this metalloproteinase. In line with this, MMP13 has been shown to be downregulated in late-stage human OA cartilage[29].

The finding that over-expression of miR-9, miR-98 and miR-146 reduced the IL-1β mediated production of TNF-α and that over-expression of miR-9 reduced basal and IL-1β induced MMP13 protein release suggests that these miRNA may play a protective role in OA disease. Therefore, the observation that miR-9 and miR-98 are both upregulated in OA cartilage and OA bone tissue might suggest that their expression is triggered as part of a negative-feedback mechanism or in response to activated pro-inflammatory pathways. Neither miR-9 nor miR-98 have previously been implicated in mediating inflammatory processes. AREG was predicted to be targeted by both these miRNAs, which as stated above were both upregulated in OA tissue. Interestingly, AREG has recently been proposed as a pro-inflammatory mediator in the pathogenesis of rheumatoid arthritis (RA), with elevated levels detected in the synovial fluid in RA compared to OA patients[30] Hence miR-9 and miR-98 may be inhibiting AREG function in the context of OA, but at present this is speculative, since direct binding of these miRNAs to AREG needs to be assessed.

Recent studies have suggested a role for miR-9 in neuronal development of the spinal cord[31], neuronal dysfunction in Alzheimer's disease[32] and insulin release from pancreatic cells[33], whilst miR-98 expression has been associated with head and neck cancers[34].

One of the most interesting findings of the pathway analysis was the significant proportion of miR-146 predicted gene targets that mapped to the NFκB pathway, suggesting miR-146 plays a role in mediating NFκB signalling. Although further work is required to fully validate the role of mir-146 in NFκB signalling it is of interest that two predicted gene targets of miR-146, namely IRAK1 and TRAF6, are key adaptor molecules in toll-like receptor and IL-1 receptor signalling cascades that are thought mediate activation of NFκB and AP-1 pathways. Indeed both IRAK1 and TRAF6 have now been shown experimentally to be direct targets of miR-146[19], which strengthens the suggestion from our bioinformatics analysis that miR-146 is involved in the NFκB signalling pathway.

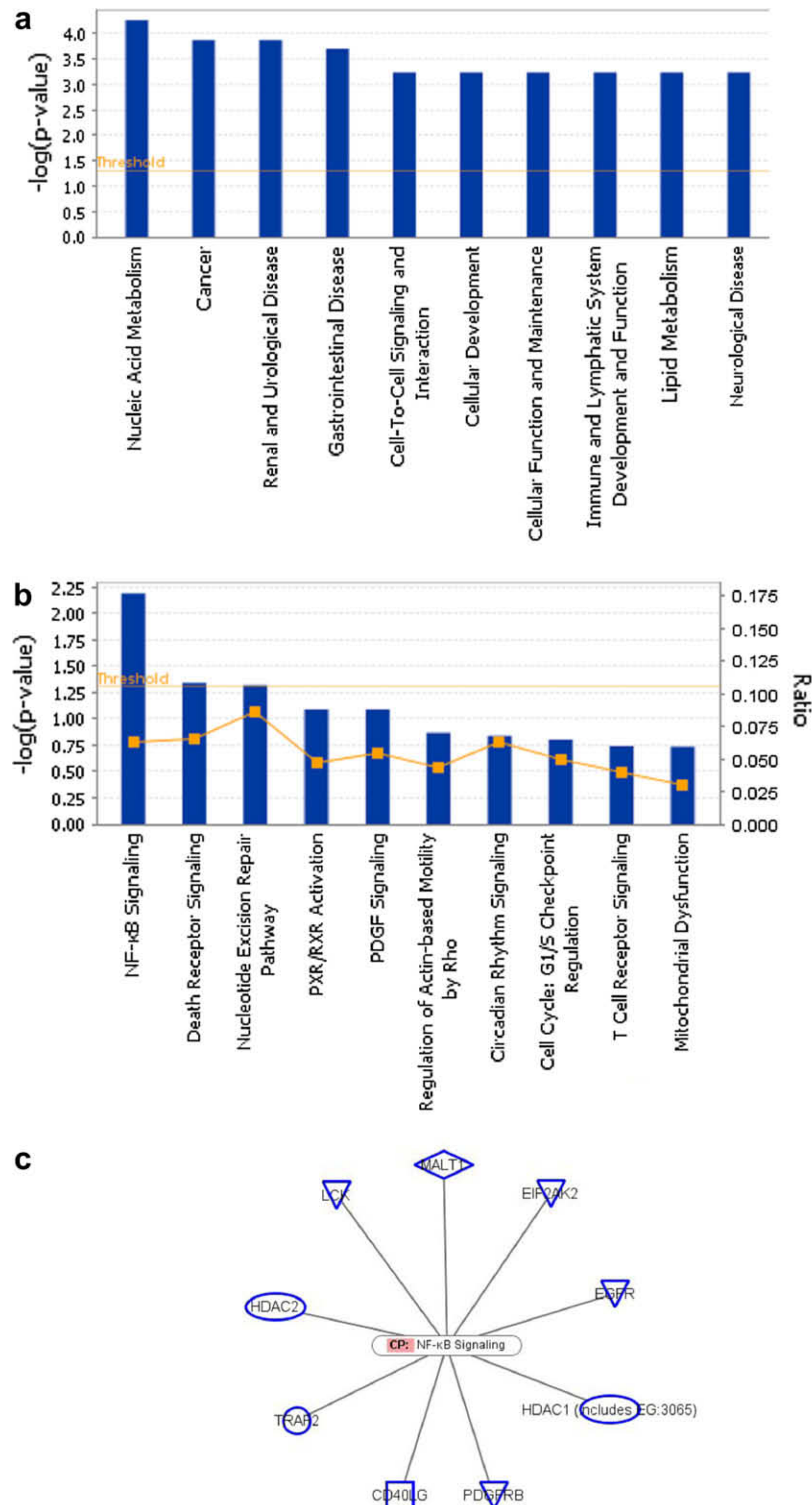

Fig. 4. Pathway analysis of miR-146. Core functional pathway analysis using Ingenuity Pathway Analysis application of the top 200 predicted gene targets of miR-146. (**a**) Most significant biological functions mapped to the network of miR-146 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3 using Fisher's exact test. (**b**) Most significant canonical signalling pathways from the Ingenuity canonical pathway library mapped to the network of miR-146 gene targets. Threshold bar shows cut-off point of significance $P < 0.05$, $-\log(P\text{-value})$ of 1.3 using Fisher's exact test. Line shows ratio of genes in network to total number of genes in canonical pathway. (**c**) Network of miR-146 gene targets linked to the NFκB signalling pathway.

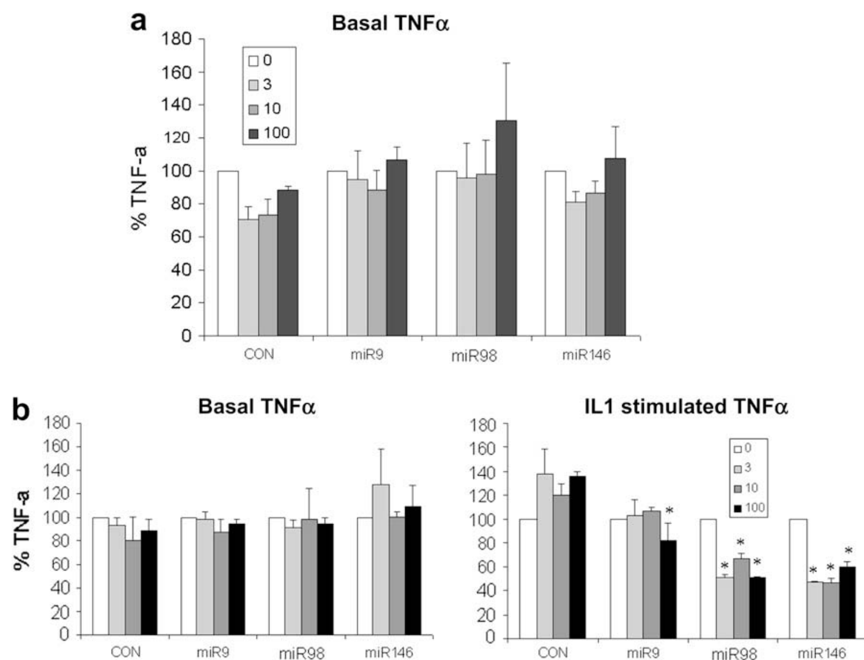

Fig. 5. MiR-9, miR-98 and miR-146 decrease IL-1 induced TNF-α secretion by human chondrocytes (**a**) The effect of inhibition of miR-9, miR-98 and miR-146 using LNA inhibitors on basal TNF-α protein production in human primary OA chondrocytes. Bars show mean % TNF-α protein ± S.E.M. ($n = 3$) relative to media control. (**b**) The effect of over-expression of miR-9, miR-98 and miR-146 on the basal and IL-1β-induced TNF-α protein. Bars represent mean % TNF-α protein ± S.E.M. ($n = 3$) relative to media control. * = $P < 0.05$, significantly different from corresponding control value.

Importantly, it has recently been reported that LPS-mediated elevation of miR-146 in THP1 cells occurs in an NFκB dependent manner[19], which opens up the possibility that miR-146 is induced as a negative feedback regulator of inflammation. MiR-146 has recently been detected at higher levels in the synovium from rheumatoid arthritis patients compared to OA patients[35]. Our findings show that miR-146 is downregulated in late-stage OA cartilage, while over-expression negatively effects TNF-α levels suggest that reduced miR-146 expression in OA cartilage could be a factor in the promotion of an inflammatory OA state, at least in the latter stages of the disease. Mir-146 is not predicted to directly target TNF-α and indeed over-expression of miR-146 did not affect basal TNF-α secretion from chondrocytes. However, the fact that IL-1β-induced production of TNF-α was significantly reduced by miR-146 over-expression

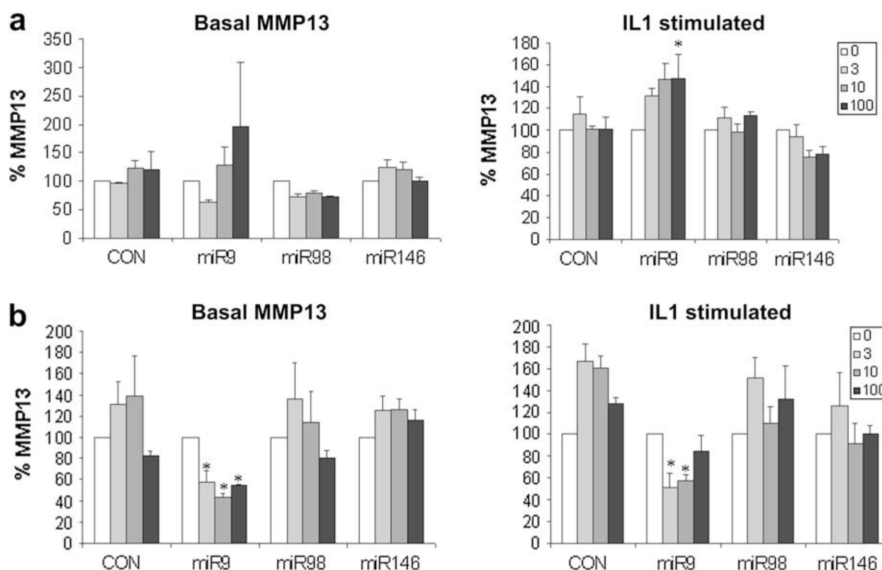

Fig. 6. MiR-9, but not miR-98 nor miR-146 decreases MMP13 secretion by human chondrocytes (**a**) The effect of inhibition of miR-9, miR-98 and miR-146 using LNA inhibitors on basal and IL-1β-induced MMP13 protein production in human primary OA chondrocytes. Bars show mean % MMP13 protein ± S.E.M. (n=3). * = $P < 0.05$, significantly different from corresponding LNA control inhibitor value. (**b**) The effect of over-expression of miR-9, miR-98 and miR-146 on basal and IL-1β-induced MMP13 protein in primary human OA chondrocytes. Bars represent mean % MMP13 protein ± S.E.M. ($n = 3$). * = $P < 0.05$, significantly different from corresponding control value.

could conceivably be due to direct targeting of IL-1 receptor-associated kinase 1 (IRAK1) - a known miR-146 target[19].

Current approaches to developing disease-modifying drugs in OA have focussed on inhibition of the MMPs (e.g MMP13) or modulating IL-1β either directly or *via* signal transduction (e.g prostaglandin J2). Understanding the regulation of miRNAs or directly targeting groups of key miRNA identified as playing a role in the disease process may be a valid alternative therapeutic approach. Indeed, a small number of miRNA therapies are being explored in cancer, viral infection, metabolic disorders and inflammatory disease. In considering whether particular miRNA have value either as therapeutics it is important to consider their species conservation in order that *in vivo* efficacy against biomarkers of disease can be demonstrated. Analysis of miR-146 transcripts using the Sanger registry shows that miR-146 is evolutionary well conserved, with close homology between rodent (for which there are established OA models) and human miR-146.

In summary, given the role of miRNA in mediating the translation of target mRNA into protein, the identification of differentially expressed miRNA in OA tissue we report here could have important diagnostic and therapeutic potential. The ability to selectively control aberrant protein expression through miRNA could provide a novel means to treat OA disease, whilst the differentially expressed miRNAs we have identified could prove to be useful diagnostic "at-risk" biomarkers of OA disease progression.

Conflict of interest

There are no conflicts of interest from any of the authors of this manuscript that could have inappropriately influenced this work.

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