

## Detecting new microRNAs in human osteoarthritic chondrocytes identifies miR-3085 as a human, chondrocyte-selective, microRNA



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### ARTICLE INFO

#### Article history:

Received 20 May 2015

Accepted 12 October 2015

#### Keywords:

Osteoarthritis

Cartilage

microRNA

Integrin

### SUMMARY

**Objective:** To use deep sequencing to identify novel microRNAs (miRNAs) in human osteoarthritic cartilage which have a functional role in chondrocyte phenotype or function.

**Design:** A small RNA library was prepared from human osteoarthritic primary chondrocytes using in-house adaptors and analysed by Illumina sequencing. Novel candidate miRNAs were validated by northern blot and qRT-PCR. Expression was measured in cartilage models. Targets of novel candidates were identified by microarray and computational analysis, validated using 3'-UTR-luciferase reporter plasmids. Protein levels were assessed by western blot and functional analysis by cell adhesion.

**Results:** We identified 990 known miRNAs and 1621 potential novel miRNAs in human osteoarthritic chondrocytes, 60 of the latter were expressed in all samples assayed. MicroRNA-140-3p was the most highly expressed microRNA in osteoarthritic cartilage. Sixteen novel candidate miRNAs were analysed further, of which six remained after northern blot analysis. Three novel miRNAs were regulated across models of chondrogenesis, chondrocyte differentiation or cartilage injury. One sequence (novel #11), annotated in rodents as microRNA-3085-3p, was preferentially expressed in cartilage, dependent on chondrocyte differentiation and, in man, is located in an intron of the cartilage-expressed gene *CRTAC-1*. This microRNA was shown to target the *ITGA5* gene directly (which encodes integrin alpha5) and inhibited adhesion to fibronectin (dependent on alpha5beta1 integrin).

**Conclusion:** Deep sequencing has uncovered many potential microRNA candidates expressed in human cartilage. At least three of these show potential functional interest in cartilage homeostasis and osteoarthritis (OA). Particularly, novel #11 (microRNA-3085-3p) which has been identified for the first time in man.

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### Introduction

Osteoarthritis (OA) is a degenerative joint disease characterised by degradation of articular cartilage as well as thickening of subchondral bone and formation of osteophytes at the joint margin<sup>1</sup>. The chondrocyte is the only cell in cartilage, is crucial to tissue

function<sup>2</sup> and must express appropriate genes to achieve tissue homeostasis; this is altered in OA<sup>3</sup>.

Small non-coding RNAs, microRNAs (miRNAs), are important regulators of gene expression in human cells. miRNAs are transcribed as primary transcripts and processed to short stem-loop structures (pre-miRNA) in the nucleus. The pre-miRNA is processed by Dicer, forming two complementary short RNA molecules one of which (the guide strand) is integrated into the RNA-induced silencing complex (RISC), the other of which (the passenger strand or miRNA\*) is degraded<sup>4</sup>. After integration into RISC, miRNAs base pair with their mRNA targets, usually in the 3'UTR, but sometimes in promoter or coding regions. Depending on the level of sequence

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complementarity between miRNA and target mRNA, RISC either cleaves the target mRNA (if complementarity is high) or suppresses translation and mainly promotes more rapid mRNA degradation (if complementarity is lower) the latter being predominant in animals<sup>5,6</sup>.

Many miRNAs are regulated during cartilage development, including by the cartilage-specifying transcription factor Sox9 (e.g., miR-140 and miR-455)<sup>(7,8)</sup>, Barter *et al.*, unpublished), or regulating Sox9 expression (e.g., miR-675 and miR-145)<sup>9,10</sup>. Many miRNAs are expressed differentially during OA<sup>11</sup>, though with high variability. These include miR-9, miR-98, miR-146a<sup>12,13</sup>, miR-483, miR-149, miR-582, miR-1227, miR-634, miR-576, miR-641<sup>14</sup> and miR-27a and b<sup>15</sup>, though miRNA-140 is the most studied to date. MicroRNA-140 null mice are predisposed to the development of age-related OA-like changes<sup>16,17</sup> and increased cartilage destruction in surgically-induced OA. Conversely, in an antigen-induced arthritis model, transgenic over-expression of miR-140 in chondrocytes protected against cartilage damage<sup>16</sup>.

There are 1881 precursors and 2588 mature human miRNAs in Release 21 of miRBase (<http://www.mirbase.org>). Deep sequencing has been used to identify potential new miRNAs in a number of cells and tissues, including rat cartilage during development<sup>18</sup>, however, this has not been applied to human chondrocytes to date. In this study, we aimed to use deep sequencing to identify novel miRNAs in human osteoarthritic cartilage and identify their possible functional roles. Osteoarthritic cartilage was used to reveal the maximum number of disease-associated miRNAs. We sequenced libraries of small RNAs purified from human osteoarthritic chondrocytes using a recently developed approach<sup>19</sup>. Potential novel miRNAs were then validated and characterised further. Of these, miRNA-3085, previously annotated in the mouse and rat, was identified as a human miRNA, selectively expressed in cartilage, which affects chondrocyte function via decreased expression of integrin alpha5.

## Method

### Cell culture

SW1353 chondrosarcoma cells were from American Type Culture Collection<sup>8</sup>. Primary human articular chondrocytes (HACs) were isolated from osteoarthritic cartilage<sup>20</sup>. DF1 cells (a gift from Prof. Andrea Münsterberg, University of East Anglia, UK) are spontaneously transformed chicken fibroblasts<sup>21</sup>. Parental and DLD-1 Dicer null cell lines were from Horizon Discovery (Cambridge UK) and originated from a colorectal adenocarcinoma.

### Tissue collection

Femoral head and knee cartilage were obtained from OA [HOA (Hip OA) age 61–89 years, 3M, 3F; KOA (Knee OA) age 55–71, 2F, 3M] and trauma [NOF (neck of femur), age 71–92 years, 3F, 3M] patients undergoing total joint replacement surgery at the Norfolk and Norwich University Hospital, UK. OA was diagnosed using clinical history, examination and X-ray; confirmation of gross pathology was made at time of joint removal. Fracture patients had no known history of joint disease and cartilage was free of lesions. This study was performed with Ethical Committee approval and all patients provided informed consent. Cartilage was dissected and snap frozen in liquid nitrogen within 30 min of surgery.

### RNA purification and quantitative real time PCR (qRT-PCR)

RNA was purified from cartilage or HACs using mirVana™ miRNA Isolation Kit (Life Technologies). For mRNA analysis by qRT-PCR from cultured cells, cDNA synthesis used the 'Cells-to-cDNA'

method as described<sup>20</sup>. For direct reverse transcription from purified RNA, Superscript II and qRT-PCR for mRNA expression was performed as described<sup>8</sup>. Primer sequences are listed in [Supplementary Table 1](#). Primers to measure novel miRNAs were designed using the Exiqon web-based assay tool, sequences for these and primers for known miRNAs are proprietary.

### Northern blot

RNA was separated on a 12% (w/v) polyacrylamide gel and transferred to Hybond-NX membrane (Amersham Biosciences). The blot was hybridised in ULTRAHyb-Oligo buffer (Life Technologies) with a  $\gamma$ ATP-labelled probe complementary to the miRNA at 37°C overnight. Membranes were exposed to a Kodak Phosphor Screen SD230 and scanned on a Molecular Imager FX reader (Bio-Rad) for quantification.

### Next generation sequencing

Total RNA was extracted from primary HACs and small RNAs enriched from 10  $\mu$ g total RNA using the mirVana™ miRNA Isolation Kit. The small RNA library was prepared using the Illumina Small RNA V1.5 Sample Preparation Guide, however sRNA adaptors were substituted with High Definition (HD) adaptors<sup>19</sup>. Approximately 200 ng RNA enriched for small RNA was ligated to adenylated 3' HD adaptor with truncated T4 RNA ligase 2 (New England Biolabs). The ligated fragment was then ligated to 5' HD adaptor using T4 RNA ligase 1 (New England Biolabs). The ligated fragment was reverse transcribed followed by PCR amplification and size fractionated on an 8% (w/v) PAGE gel. A band corresponding to 145–150bp was gel purified and analysed on an Illumina Genome Analyzer IIX with 50 nt read length (Baseclear, Netherlands). Reads were trimmed for 4 nt barcodes on both ends and for Illumina adaptors on the 3' end. Resulting reads longer than 16 nt were mapped to the human genome (version GRCh38) using Patman software<sup>22</sup>, no mismatches were allowed. Reads mapping to more than 100 loci were discarded. The remaining reads were inputted to miRCat<sup>23</sup> with default parameters. miRCat novel miRNA candidates were separated from known miRNAs using in-house scripts.

### Transfection with siRNA

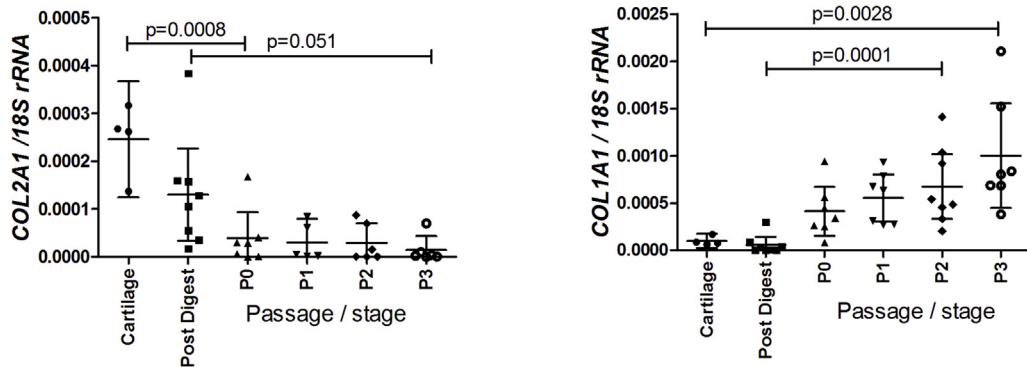
Primary HACs were plated at  $2.5 \times 10^5$  cells/well of a 6-well tissue culture plate and incubated overnight in complete medium. Cells were transfected in serum- and antibiotic-free DMEM using Lipofectamine 2000 (Invitrogen), miRNA mimics at 30 nM (Qiagen), miRNA inhibitors at 50 nM (Qiagen), or non-targeting controls (All Stars at 30 nM (Qiagen), miScript Inhibitor control at 50 nM (Qiagen)), or mock transfection. Cells were incubated for 6 h in serum-free and antibiotic-free media. Media was replaced with complete medium for a further 48 h.

For functional analysis, RNA (pooled from three samples per condition) was subjected to whole genome array using the Illumina Human HT12v4 platform (Source Bioscience). Whole genome array was normalised using R with the Lumi package<sup>24</sup>, background correction and normalisation used a between-array quantile methodology. Normalised data were analysed to measure fold-change expression. Target sequences for novel candidate miRNAs were identified using R with the Biostrings 2.28.0 package in 3'UTRs of the human genome.

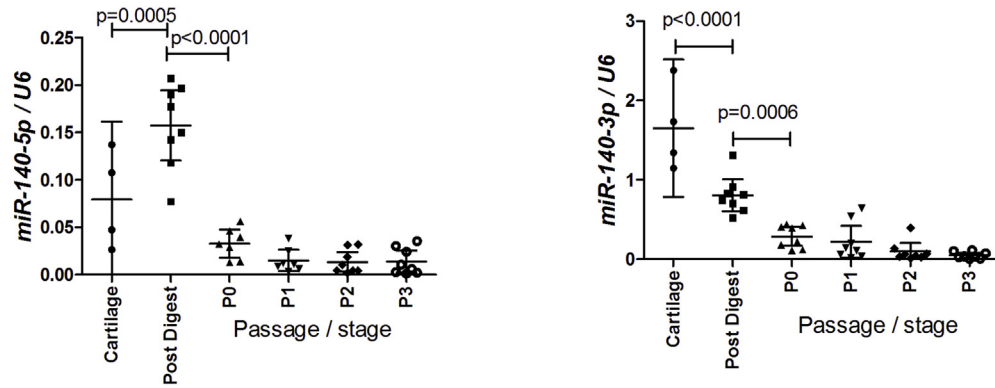
### Chondrogenic differentiation

Human mesenchymal stem cells (hMSC) were resuspended in chondrogenic culture medium consisting of high glucose

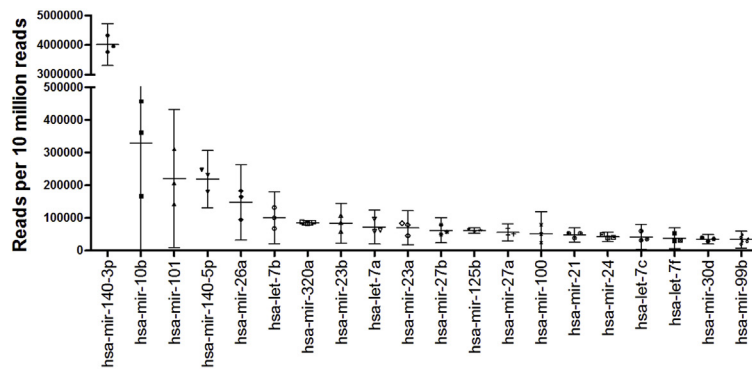
**A**



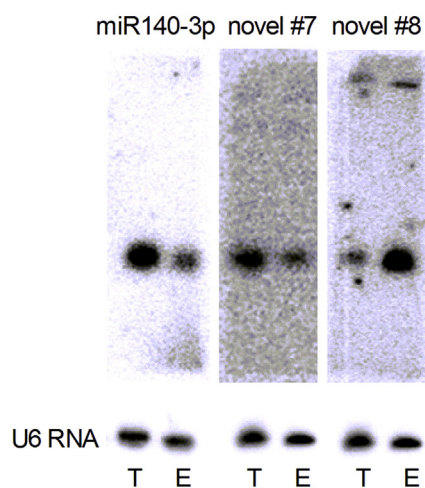
**B**



**C**



**D**



Dulbecco's modified Eagle's medium containing 100 µg/ml sodium pyruvate (Lonza), 10 ng/ml TGF-β3 (Peprotech), 100 nM dexamethasone, 1× ITS-1 premix, 40 µg/ml proline, and 25 µg/ml ascorbate-2-phosphate (all from Sigma–Aldrich, Poole, UK).  $5 \times 10^5$  hMSC in 100 µl medium were pipetted onto 6.5 mm diameter, 0.4-µm pore size polycarbonate Transwell filters (Merck Millipore), centrifuged in a 24-well plate (200 g, 5 min), then 0.5 ml of chondrogenic medium was added to the lower well as described<sup>25</sup>. Media were replaced every 2 or 3 days up to 14 days.

#### Mouse femoral head cartilage wounding assay

A mouse femoral head cartilage wounding assay was as previously described<sup>26</sup>. Briefly, the acetabulofemoral joint was exposed, the hip joint dislocated and the femoral cap was avulsed using forceps. Tissue was cultured in serum-free medium and harvested into 500 µl Trizol (Life Technologies) at the time points: 0, 1, 3, 6, 12, 24 and 48 h and stored at –80°C. Samples were homogenized with a disposable pestle, insoluble material removed by centrifugation and RNA purified as manufacturer's instructions.

#### Transient transfection

The 3' UTR of mRNAs was amplified by PCR (using primers in [Supplementary Table 1](#)) and subcloned into the pmirGLO Dual-Luciferase Vector (Promega). Mutation of the miRNA seed sequence used QuikChange (Agilent). Constructs were sequence verified. DF1 cells were plated into a 96 well plate at  $3.75 \times 10^4$  cells/cm<sup>2</sup> overnight, transfected with 100 ng luciferase reporter plasmid, 50 nM miRNA mimic, inhibitor or control using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen), and incubated for 48 h. Cell lysates were assayed using the Dual Luciferase Reporter Assay Kit (Promega) and luciferase was normalised to renilla to control for the transfection efficiency.

#### Western blot

Whole cell lysates were harvested into ice cold RIPA buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 1% (v/v) Triton x-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1x protease cocktail III tablet (Fisher Scientific)). Samples were separated on reducing SDS-PAGE, transferred to PVDF membrane and probed overnight at 4°C. The anti-integrin alpha5 antibody (Abcam ab150361) was detected using HRP-conjugated secondary antibodies (DAKO), visualised using LumiGLO reagent (New England Biolabs) and exposed to Kodak Biomax MS film (Sigma–Aldrich).

#### Cell adhesion

96-well plates were coated overnight with 10 µg/ml fibronectin (Sigma–Aldrich) at 4°C and blocked with 1% (w/v) BSA. SW1353 cells were transfected with 100 nM miRNA mimic, siRNA against ITGA5 or All Stars non-targetting control for 48 h as above. Cells were trypsinised and resuspended in serum-free medium at

$4 \times 10^5$  cells/ml. Antibodies (anti-alpha5 integrin, Abcam ab25251 or control IgG) were added to cell suspension at 10 µg/ml and pre-incubated for 10 min at 37°C. Cells were added at 100 µl per well in at least triplicate for 15 min. Cells were fixed with 4% (v/v) paraformaldehyde and stained with 1% (w/v) methylene blue in 10 mM sodium borate. Cells were lysed with 50% (v/v) ethanol in 0.1M HCl for 15 min and absorbance measured at 590 nm.

#### Statistics

Data are plotted as mean with 95% confidence intervals. Data were tested for normality prior to further analysis using Student's *t*-test to compare between two samples, or one-way ANOVA with post-hoc Tukey's test to compare between multiple samples. All statistical analyses were performed using GraphPad Prism version 5 or PASW Statistics 18.

## Results

#### Purification of RNA

We sought to identify optimal samples for sequencing to detect all cartilage miRNAs. Total RNA was purified from articular cartilage taken at total knee replacement for OA, chondrocytes immediately after tissue digestion and cells across passage in monolayer culture. [Fig. 1\(A\)](#) shows that *COL2A1* expression decreased and *COL1A1* increased with chondrocyte isolation and culture as expected. MicroRNA-140-5p increased in expression (~2-fold, *p* = 0.0005) in chondrocytes digested from cartilage [[Fig. 1\(B\)](#)], then decreased in monolayer. Interestingly, miR-140-3p was highest in cartilage and then decreased across isolation and passage [[Fig. 1\(B\)](#)]. Since miR-140-5p is strongly implicated in OA, we used RNA isolated from cells digested from cartilage (which also gives the highest quality RNA) as the starting point for sequencing studies.

#### Small RNA high-throughput sequencing

cDNA libraries were generated from small RNA isolated from chondrocytes taken from three independent osteoarthritic knees. High definition adaptors were used (see Methods) to reduce bias and reveal maximum sequence<sup>19</sup>. Approximately 20 million sequencing reads were obtained per sample and analysed using miRcat<sup>23</sup>. Known miRNAs were confirmed using miRBase (Release 20, [www.mirbase.org/](http://www.mirbase.org/)<sup>27</sup>) with 990 individual known miRNAs within the dataset, of which 630 were present in all three patients. The 20 miRNAs with highest read number per 10<sup>7</sup> sequencing reads are shown in [Fig. 1\(C\)](#). Surprisingly, the greatest number of sequencing reads came from miR-140-3p, the so-called passenger strand of miR-140 and investigation of its function is the subject to another manuscript (Wheeler *et al.* in preparation). Most of the top 20 miRNAs have previously been linked with cartilage or arthritis, apart from miR-23a, miR-100 and miR-99a<sup>11</sup>.

MiRcat generated 60 candidate novel miRNAs in all three samples ([Supplementary Table 2](#)). These 60 candidates were

**Fig. 1.** Gene expression in articular chondrocyte isolation and expansion. Expression levels of (A) *COL2A*, *COL1A1*, (B) miR-140-5p, miR-140-3p were measured by qRT-PCR from RNA isolated from: osteoarthritic knee cartilage tissue, isolated chondrocytes (post digest) and subsequent P0, P1, P2, and P3 passaged chondrocytes from monolayer culture. Data were normalised to 18S rRNA (mRNA) or U6 RNA (miRNA) expression. (RNA was obtained from 8 patients for chondrocytes and 4 patients for tissue; a one way Anova analysis with a post hoc Tukey test was used to test for significance; data are plotted as mean ± 95% confidence interval. (C) Deep sequencing of a small RNA library of post-digest chondrocyte RNA showed 630 known miRNA in all three samples sequenced, the 20 miRNA with the highest read numbers are shown; *n* = 3, data are plotted as mean ± 95% confidence interval. (D) Expression of candidate novel miRNA #7 and #8 was measured by northern blot using 10 µg SW1353 total (T) RNA or 2 µg of RNA enriched (E) for small RNAs. MicroRNA-140-3p was used as a size control and the small RNA U6 was used as a standard reference for the northern blot.

further selected (using the presence of both miRNA strands, number of genomic locations; read number; level in Dicer 1 null cells), reducing the number of candidate miRNAs to 16.

#### Measurement of candidate miRNAs

Initially, candidate miRNAs were measured by northern blot in total RNA purified from SW1353 chondrosarcoma cells. A number of novel candidates gave bands at high molecular weight, larger than miRNAs (data not shown; novel #1, #3, #4, #5, #9, #12, #13, #15, #16). Novel #7 and #8 gave an appropriately sized band similar to miR-140-3p [Fig. 1(D)]. Novel #6 gave multiple small bands. Novel #2, #10, #11 and #14 gave no signal (data not shown) and were included in further analyses.

Incorrect size on northern therefore triaged 10 miRNAs. The six remaining candidate novel miRNAs, including those with no detectable signal on northern blot, were measured by qRT-PCR in hip cartilage from OA patients and patients fracturing their neck of femur (NOF) [novel #2, #7 and #11, miR-140 and miR-455 shown in Fig. 2(A)–(D)]. No candidate novel miRNAs showed a significant difference between hip OA cartilage compared to NOF. MicroRNA-140-5p showed a trend to increased expression ( $p = 0.0587$ ) in OA cartilage compared to NOF as we have previously reported<sup>8</sup>; the more highly expressed miR-140-3p showed no difference between groups.

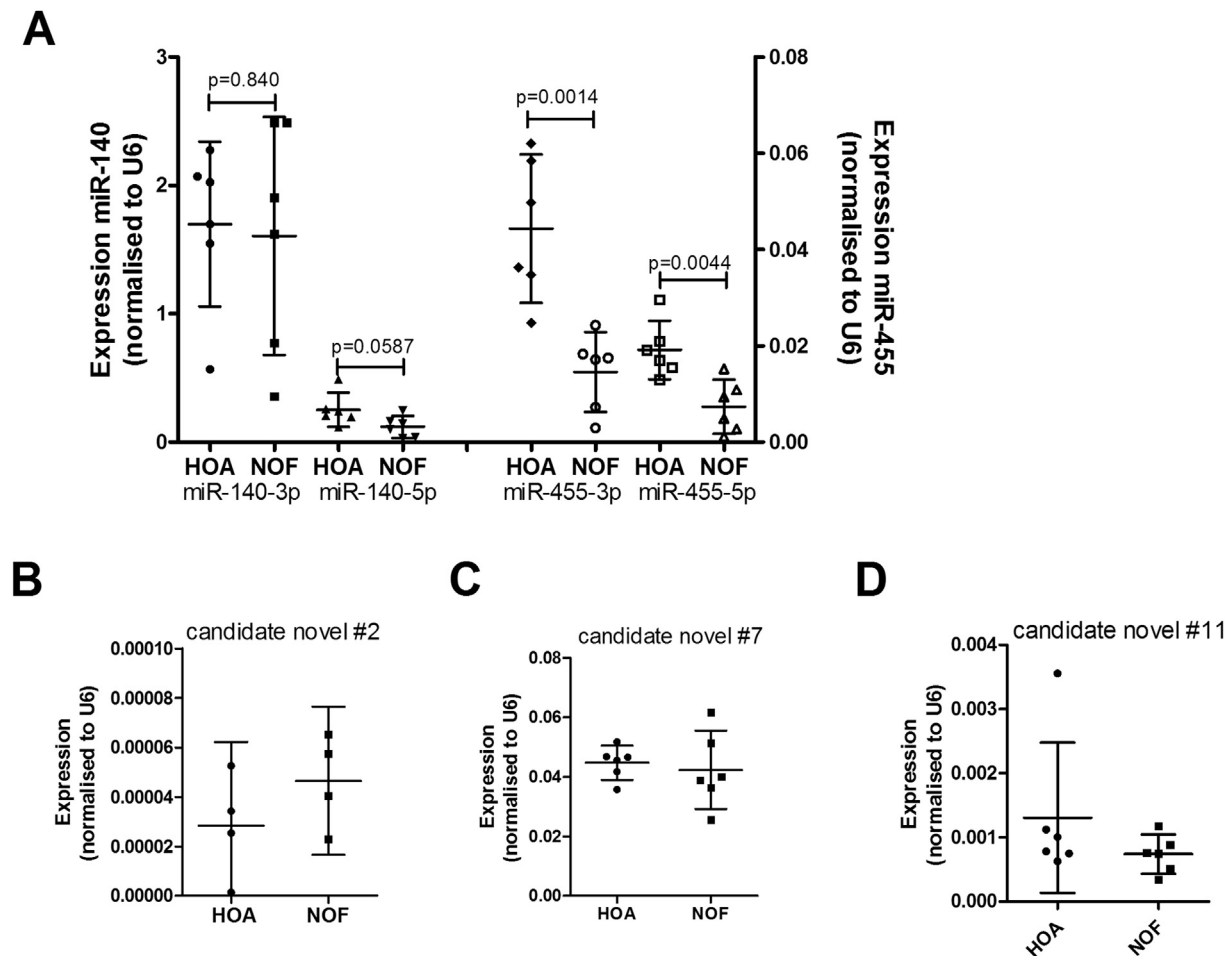
During the course of this project, a number of our novel miRNAs were annotated on miRBase. Novel #2, hsa-miR-6509-5p; #7, hsa-miR-664b-3p; #8 is a truncated form of hsa-miR-1277-5p; #10, hsa-miR-487a-5p; #11, mmu/rno-miR-3085-3p (though not annotated as a human miRNA). Since none of these have been further characterised, they remained in downstream analyses.

All novel miRNAs were measured in articular cartilage and across human tissue panels. Novel #11 showed selectivity of expression in cartilage compared to any other tissue [Fig. 3(A)]. This was equivalent to miRNA-140-5p [Fig. 3(B)] or miR-140-3p (data not shown) and similar to miRNA-455-3p or miR-455-5p [Fig. 3(C)] though this miRNA showed strong expression in cervix. Expression patterns of the other candidates were widespread and not predominant in cartilage (data not shown).

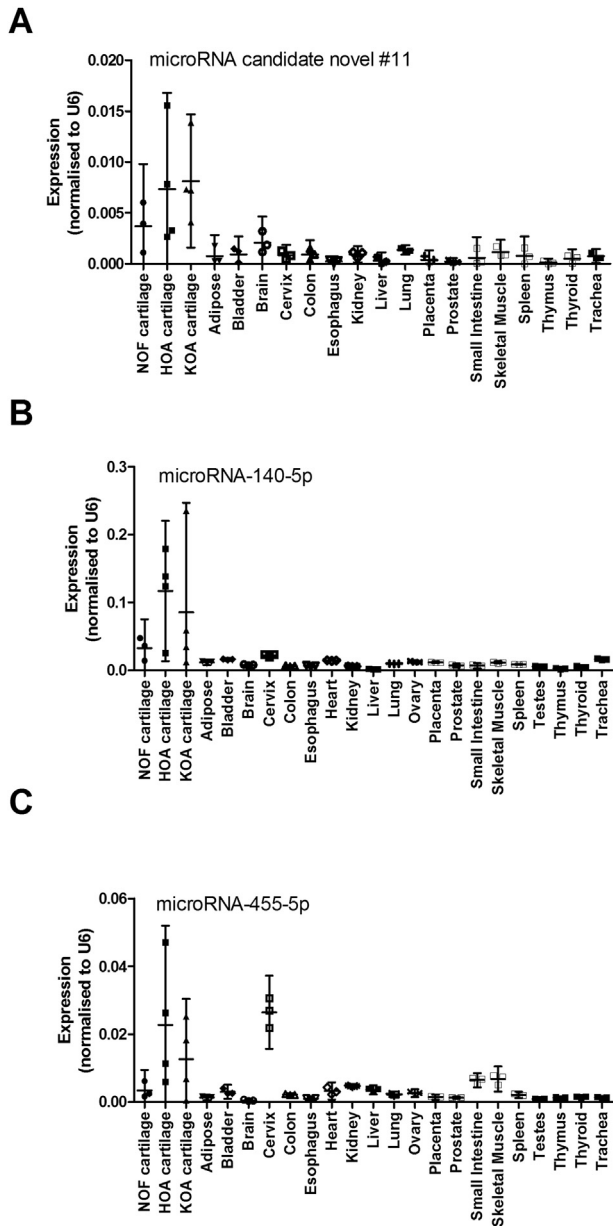
#### Further characterisation of candidate miRNAs

Three miRNAs, novel #2, #7 and #11, were all expressed at a statistically significantly lower level in a Dicer null cell line (DLD-1) than in an isogenic wild type control line (data not shown), and these were analysed further.

Fig. 4(A) shows that novels #7 and #11 have significantly higher expression in cartilage tissue than extracted cells, with further decrease in monolayer or with increasing passage. Novel #2 is not modulated by cell extraction or culture (data not shown).



**Fig. 2.** MicroRNA expression in cartilage tissue from hip osteoarthritis (HOA) and hip cartilage following fracture to the neck of femur (NOF). Expression levels of (A) miR-140, miR-455, (B) candidate novel miRNA #2, (C) candidate novel miRNA #7, (D) candidate novel miRNA #11 were obtained by qRT-PCR from RNA isolated from cartilage tissue. Data were normalised to U6 RNA expression. Graphs show mean  $\pm$  95% confidence interval.  $n = 6$ , analysed by Student's  $t$ -test.



**Fig. 3.** MicroRNA expression across a human tissue panel. Expression levels of miRNAs (A) candidate novel miRNA #11, (B) miRNA-140-5p, (C) miRNA-455-5p were obtained by qRT-PCR from RNA isolated from 23 human tissues as shown. NOF, hip cartilage following fracture to the neck of femur; HOA, hip osteoarthritis; KOA, knee osteoarthritis. Data were normalised to U6 RNA expression. RNA of all cartilage samples was from 3 patients. RNA from all remaining tissues was from a pool of three patients with  $n = 3$  technical replicates. Data are plotted as mean  $\pm$  95% confidence interval.

In an assay of cartilage formation from human bone-marrow derived MSC, expression of novel #2 increased at day 7, but decreased to later time points [Fig. 4(B)], similarly to Sox9<sup>25</sup>. Novel #7 increased throughout cartilage formation [Fig. 4(B)], more similarly to COL2A1<sup>25</sup>. Novel #11 showed no significant change across the 14 days (data not shown).

In the mouse hip avulsion assay of cartilage wounding, novel #7 increased in expression across 48 h, whilst candidate #11 was expressed in a biphasic pattern, with two peaks across 48 h [Fig. 4(C)]. Novel #2 is not found in the mouse genome. MicroRNA-140 (both strands) and miR-455 (both strands) increased in expression across the time course similarly to novel #7 (data not shown).

### *ITGA5* is a direct target of novel #11 (miR-3085-3p)

Primary HACs (from three separate patients) were transfected with either miRNA mimics or inhibitors for novels #2, #7 and #11 or negative controls for 48 h, followed by microarray. Genes which are true targets of the miRNA will decrease in expression in cells treated with miRNA mimics, increase in expression in cells treated with miRNA inhibitors and contain a seed sequence for the miRNA in their 3'UTR. Supplementary Table 3(A)–(C) show the top genes for each candidate which fit these criteria.

Since novel #11 was expressed in a cartilage-selective manner, and qRT-PCR verified *ITGA5* as a potential target (data not shown), this gene was further assessed. Fig. 5(A) shows that expression of luciferase controlled by the 3'UTR of *ITGA5* was decreased by novel #11 and that this effect was abrogated by mutation of the seed sites (from AGCCAG to GAGCTC). This demonstrates that novel #11 directly targets the *ITGA5* gene. Western blot shows that the level of integrin alpha5 (encoded by *ITGA5*) is reduced by transfection with the novel #11 miRNA mimic [Fig. 5(B)], with the same bands reduced by an *ITGA5* targeting siRNA. The functional potential of novel #11 was probed by measuring adhesion to fibronectin, mediated by integrin alpha5beta1. A function blocking antibody against integrin alpha5 reduces adhesion, as does EDTA. Transfection of cells with candidate #11 inhibited adhesion to a similar extent to siITGA5 [Fig. 5(C)].

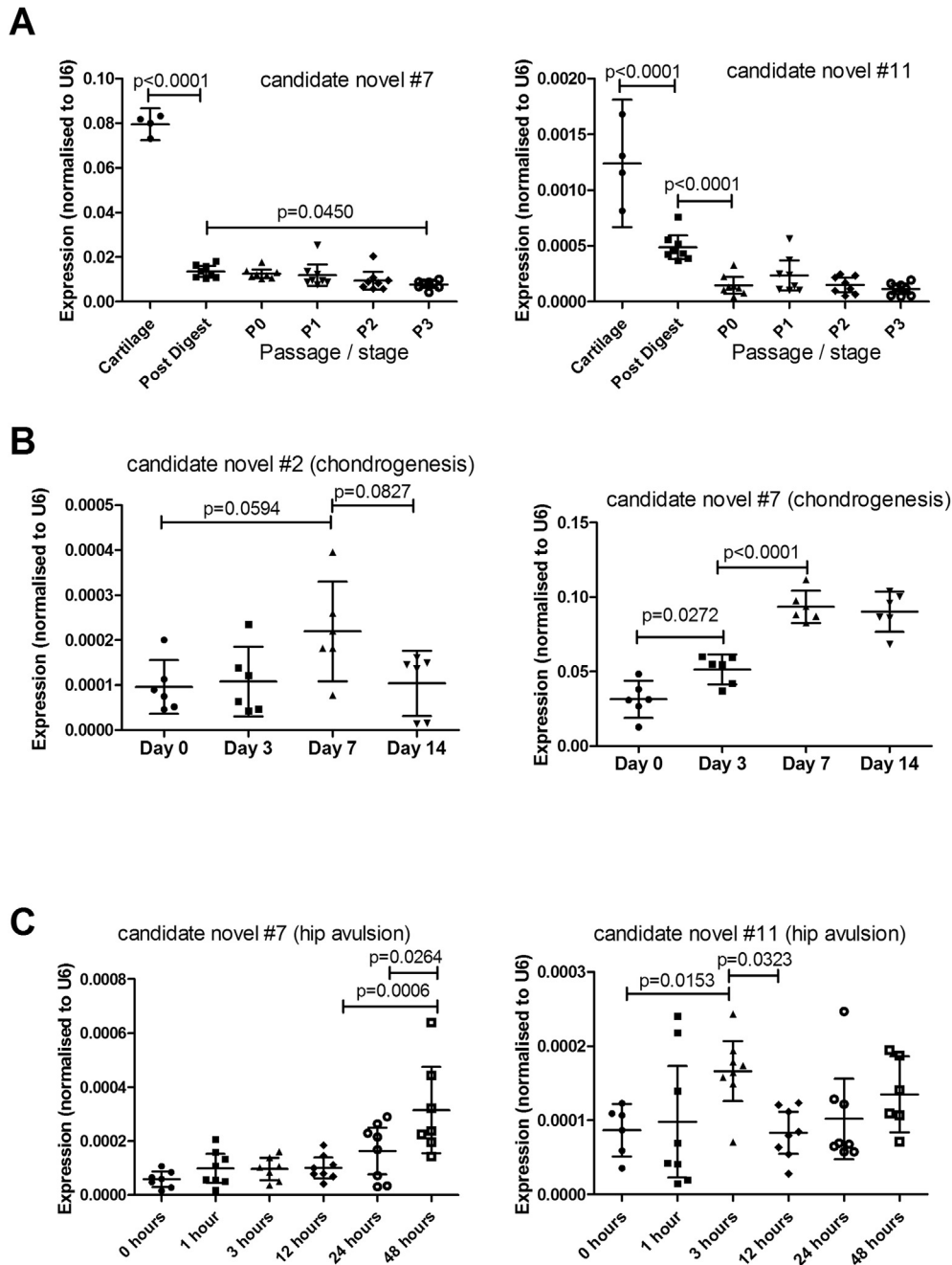
### Discussion

We initially sought to identify the best source of chondrocyte RNA from which to identify miRNAs. We measured a number of miRNA and mRNA known to be important in OA across cartilage tissue and cells derived from it across passage in culture. For miRNA-140-5p, the miRNA most implicated in cartilage homeostasis and OA, expression was highest in cells digested from cartilage rather than in the tissue itself. This increased expression of miR-140-5p may be a response to injury, a known phenomenon for miRNAs in several areas of physiology and pathology, including cartilage e.g.,<sup>28,29</sup>. Expression of both *MMP1* and *MMP13* is also significantly higher in cells digested from cartilage compared to the tissue itself (data not shown).

We sequenced libraries from three OA patients using so-called 'high definition' adaptors<sup>19</sup> which have been shown to approximately double read coverage, finding 60 potential new miRNA sequences in all three. Sun *et al.* undertook a deep sequencing analysis of rat cartilage across development and uncovered 86 novel candidate miRNAs<sup>18</sup>, however, further validation of these sequences was not reported.

The miRcat software<sup>23</sup> was used to designate miRNAs by factors including, location, abundance, secondary structure, number of mismatches between 5p and 3p strand and hairpin length. These sequences were then searched in miRBase<sup>27</sup> to discount miRNAs already known and novel sequences further triaged as described. This led to 16 potential novel miRNAs being further investigated. New releases of miRBase have included some of the candidate novel miRNAs, identified in other sequencing projects.

The levels of each miRNA using either deep sequencing, northern blot, or qRT-PCR were not comparable. It has previously been reported that measurement between platforms shows high variability<sup>30</sup>. miRNAs vastly different in read number within the sequencing library, where bias is known, showed much closer expression in northern blot analysis (e.g., miR-140, miR-455 and novel #7 or #8) or in qRT-PCR (e.g., mean Ct for miR-455-5p in hip OA cartilage = 25.11 and for novel #11 = 28.2), where Ct < 30 is robust expression.

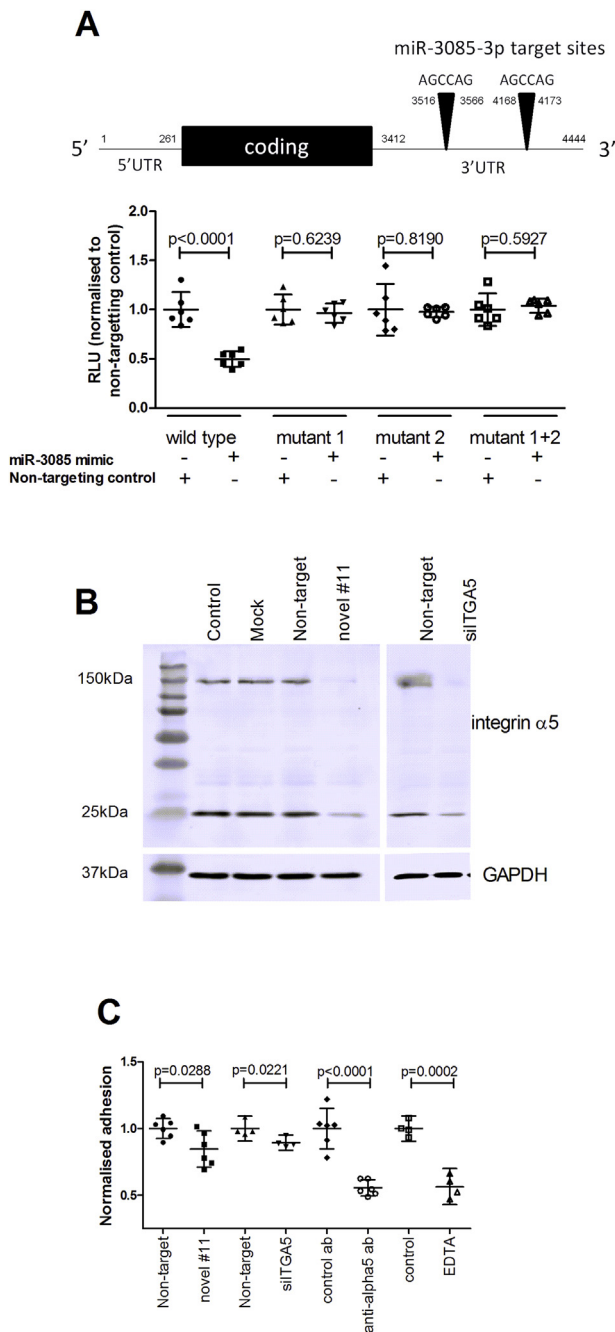


**Fig. 4.** MicroRNA expression in models of chondrocyte differentiation or injury. (A) MicroRNA expression in articular chondrocyte isolation and expansion. Expression levels of candidate miRNA novel #7 and novel #11 were measured by qRT-PCR from RNA isolated from: osteoarthritic knee cartilage tissue, isolated chondrocytes (post digest) and subsequent P0, P1, P2, and P3 chondrocytes passaged through monolayer culture. Data were normalised to U6 RNA expression. (RNA was obtained from 8 patients for chondrocytes and 4 patients for tissue; a one way Anova analysis with a post hoc Tukey test was used to test for significance; data are plotted as mean  $\pm$  95% confidence interval.). (B) MicroRNA expression in a mesenchymal stem cell chondrogenesis assay. Expression levels of candidate miRNA novel #2 and novel #7 were measured by qRT-PCR from RNA obtained from mesenchymal stem cells induced through chondrogenesis to form cartilage discs. RNA samples were taken at time points during the assay: day 0, 3, 7, 14. Data shows mean  $\pm$  95% confidence interval,  $n = 3$ , data were normalised to U6 RNA expression and analysed by a one way ANOVA with a post hoc Tukey test. (C) MicroRNA expression in a cartilage injury model. Expression levels of candidate miRNA novel #7 and novel #11 were obtained by qRT-PCR from RNA isolated from hip cartilage removed from 3 to 5 week old mice and incubated in culture medium for 48 h. RNA was isolated at the time points; 0, 1, 3, 6, 12, 24 and 48 h. Data shows mean  $\pm$  95% confidence interval,  $n = 8$ . Data were normalised to U6 RNA expression and analysed by a one way ANOVA with a post hoc Tukey test.

Northern blot showed that two candidate novel miRNAs, #7 and #8 gave an appropriately sized band and can be assigned as miRNAs; this confirms recent sequence identification for novel #7 as miR-664b-3p and novel #8 as miR-1277-5p. Four candidate novel miRNAs, #2, #10, #11 and #14, gave no signal, and may be more weakly expressed in the SW1353 chondrosarcoma cell line used.

Three of these have since been described as miRNAs in recent versions of miRBASE (#2, #10 and #11). Ten candidate novel miRNAs were discounted on the basis of their size.

Expression across a broad tissue panel showed that only novel miRNA #11 was selectively expressed in articular cartilage. This pattern was similar for miR-140, with miR-455 showing high



**Fig. 5.** Functional analysis of integrin alpha5 as a target of candidate microRNA #11. (A) The schematic shows the ITGA5 cDNA with the location of target sites numbered. Cells (DF1) were transfected with the 3'UTR of ITGA5 cloned into pmirGLO, or a construct with either or both miR-3085-3p seed sites mutated (to GAGCTC) (mutant 1, 2 or 1 + 2), with or without candidate miRNA #11 (miR-3085-3p) mimic or non-targeting control. Firefly luciferase relative light units were normalised to Renilla relative light units. Data are mean  $\pm$  95% confidence interval,  $n = 6$ , analysed by a Student's  $t$  test. (B) SW1353 cells had no treatment (control), mock transfected, or transfected with a miR-3085 mimic, a siRNA against ITGA5 (siITGA5) or non-targeting siRNA; protein was extracted and separated on 12.5% (w/v) SDS-PAGE, blotted onto PVDF and probed with an anti-integrin alpha5 antibody. The blot was stripped and re-probed with a GAPDH antibody to assess loading. (C) SW1353 cells were transfected with a miR-3085 mimic, siITGA5 or a non-targeting control (100 nM) for 48 h. Cells were pre-incubated in serum-free medium with an anti-integrin alpha5 antibody or control antibody (10  $\mu$ g/ml) or EDTA (2 mM) for 10 min if required and then allowed to adhere to fibronectin for 15 min. After washing cells were fixed and stained with methylene blue, lysed and absorbance at 590 nm measured. Data are normalised to the control and presented as mean  $\pm$  95% confidence interval,  $n = 4-6$ , analysed by a Student's  $t$  test.

expression in cervix as well as articular cartilage. Novel #11 is annotated in miRBase as miR-3085 but only in the mouse or rat. In these species, the miRNA is 3' to cartilage acidic protein 1 (*CRTAC1*, previously called *CEP-68*), however, in man, it is within a final intron of the gene, though these longer human transcripts have not yet been annotated in rodents. The miRNA is conserved in mammals (data not shown). Many intronic miRNAs are functionally related to the genes they are within<sup>31</sup>. Cartilage acidic protein 1 is a protein of unknown function, which was originally described as a marker of chondrocytes in culture, able to delineate between MSC, chondrocytes and osteoblast-like cells<sup>32</sup>. The gene is expressed in both cartilage and bone tissue, but rapidly lost from osteoblasts upon culture<sup>32</sup>. Its expression is also markedly decreased when chondrocytes are digested from cartilage, but increased again in 3D culture<sup>33</sup>. *CRTAC1* is also reported to be increased in expression in microarrays of human OA cartilage (e.g.,<sup>3,34</sup>). *CRTAC-1* protein has been identified in the plasma of patients with long-bone fracture, where it was suggested either to indicate normal callus formation and healing or concomitant joint injury<sup>35</sup>.

Novel miRNA #2 (not found in mouse) is located in an intron of the gene *WDR91* which has no known function, whilst novel #7 comes from the snoRNA *SNORA36A*. It has been shown that miRNAs can be derived from snoRNAs<sup>36</sup>, and indeed miR-140 has been described as snoRNA-like miRNA<sup>37</sup>.

The pattern of expression of each miRNA in a variety of cell models was different. Broadly, novel miRNA #7 follows the same pattern as miR-140-5p and miR-455-5p and -3p. Novel #11 decreases with chondrocyte dedifferentiation, but does not reproducibly increase in the MSC chondrogenesis model.

We used overexpression of a miRNA mimic or inhibitor in primary HACs to define direct targets of the novel miRNAs. A number of possible targets are identified bioinformatically, though these remain to be validated in future studies. Several potential targets have been implicated in cartilage and OA. For novel miRNA #2, PDGF-D is a recently discovered platelet-derived growth factor isoform, where PDGF has been shown to regulate chondrocyte proliferation<sup>38</sup>; secreted frizzled-related protein 4 (sFRP4) is a Wnt antagonist with the Wnt pathway key in cartilage homeostasis and OA<sup>39</sup>; TGFBR3 encodes betaglycan, a co-receptor for TGF $\beta$ , another pathway key in OA<sup>40</sup>. For novel miRNA #7, cartilage intermediate layer protein has been implicated in cartilage structure, but also in calcification and can interact with key growth factor pathways (e.g., TGF $\beta$  or IGF)<sup>41</sup>. Sox4 is a SoxC gene, shown to be involved in skeletogenesis, but also in Wnt signalling<sup>42</sup>. TIMP-3 is an inhibitor of cartilage-degrading proteinases<sup>43</sup>. Versican, a cartilage proteoglycan has been mooted as a biomarker, along with TIMP-3, in shoulder OA<sup>44</sup>. For novel miRNA #11 (miR-3085-3p), IL-18 and Myd88 are known to be involved in tissue inflammation<sup>45</sup> and TGFBR3 is also a predicted target for this miRNA as well as novel miRNA #2 above.

The *ITGA5* gene was shown to be a direct target of novel #11 (miR-3085-3p) and the miRNA strongly reduced the level of integrin alpha5 protein in chondrocytes. Integrin alpha5beta1 has been implicated in cartilage homeostasis and destruction<sup>46</sup>, e.g., mediating signalling through matrix fragments and mechanotransduction. Functionally, novel #11 (miR-3085-3p) was shown to inhibit chondrocyte binding to fibronectin to the same extent as a specific siRNA against ITGA5 [Fig. 5(C)]. Fibronectin is a glycoprotein found in the superficial zone of cartilage and is increased in OA; functionally it has a role in lubrication, matrix organisation and in cartilage development<sup>47-50</sup>. The impact of novel #11 (miR-3085-3p) on the reported fibronectin fragment-induced expression of *MMP13* through alpha5beta1 was measured, but this miRNA also reduces the expression of basal *MMP13*, even though this gene does not have a seed sequence for this miRNA (data not shown). This



effect is likely indirect: our microarray data show that *MMP13* expression decreases with the mimic and increases with the inhibitor of novel #11, potentially via the NFκB, STAT and retinoic acid receptor pathways which are also targeted.

In summary, we have identified and validated several miRNA candidates from human osteoarthritic cartilage. Most interestingly, novel #11 is annotated in mouse and rat as miRNA-3085-3p, but is 3' of a cartilage expressed gene *CRTAC1*. In man, longer transcripts of this gene have been annotated and it is located in the last intron of the gene. This miRNA has high expression in cartilage which decreases on chondrocyte dedifferentiation, is expressed in a cartilage-selective manner and we have shown it to target the *ITGA5* gene directly, functionally disrupting integrin alpha5. These data will allow the further functional characterisation of identified miRNAs. It is clear that a number of miRNAs function in cartilage and are dysregulated in OA. A detailed understanding of the role of miRNAs in OA will allow their targeting to prevent or slow the progression of the disease.

#### Author contributions

- Conception and design: IMC, DAY, TD
- Analysis and interpretation of the data: NC, HP, TD, IMC
- Drafting of the article: NC, IMC
- Critical revision of the article for important intellectual content: All authors
- Final approval of the article: All authors
- Statistical expertise: HP, IMC
- Obtaining of funding: IMC, TES, TD
- Collection and assembly of data: NC, TES, LL, MJB, GW, HP, STD

#### Conflict of interests

There are no conflicts of interest.

#### Acknowledgements

This work was supported by grants from Arthritis Research UK (#19321 and #19424); LL was supported by a grant from the Vietnam Ministry of Education (Project 322). We would like to thank Dr Simon Tew (University of Liverpool) for his guidance in measuring gene expression in newly extracted chondrocytes; Prof Tonia Vincent (University of Oxford) for her help and advice with the murine hip avulsion model; Dr Emma Blain (University of Cardiff) for bringing chondrocyte function of alpha5beta1 integrin to our attention; Dr Jelena Gavrilovic (University of East Anglia) for her help and advice with cell adhesion assays to assess integrin function.

#### Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.joca.2015.10.002>.

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