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Original Paper

Dual and Opposing Regulation of MMP1 and MMP13 by Both Arms of miR-675 in Human Articular Chondrocytes

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Key Words
MicroRNA • (miR) • miR-arm selection • Matrix-Metalloproteases

Abstract
Background/Aims: MicroRNAs (miRs) are transcribed as stem-loop precursors harboring two different miRs on either side of the structure. Both miRs can modulate levels of cellular transcripts based on sequence complementarity between the miR and the mRNA target. The miR of the current study, miR-675, is encoded in the H19 gene with high expression in fetal/placental tissues but low levels in most adult tissues except for skeletal muscle and articular cartilage. miR-675 has a supportive role in expression of the major collagen component of articular cartilage (COL2A1) but it is unknown which arm contributes to this effect. Objectives: To determine the active arm of miR-675 in human articular chondrocytes. To evaluate effects of overexpression of both arms of miR-675 on MMP1 and MMP13, two enzymes involved in breakdown of COL2A1. To investigate whether abundance of both arms of miR-675 is dynamic. ***Methods:*** miR-arm activity was determined by association with the AGO2 complex using immunoprecipitation with an AGO2 specific antibody. miR overexpression and inhibition was used to identify indirect downstream effects on two targets of the Matrix-Metalloprotease family, MMP1 and MMP13. Data was evaluated by qPCR and enzymatic activity assays. Early passage human articular chondrocytes (up to passage 2) obtained from cartilage from both healthy and osteoarthritis affected tissue were used. To evaluate miR-675 levels in a different model, myotube differentiation was employed. ***Results:*** We show that both arms of miR-675 have opposing effects on MMP1 and MMP13; however only one arm, miR-675-3' is active in human articular chondrocytes. We demonstrate that during myotube differentiation, high expression of both arms of miR-675 is observed as well as an increase in expression of MMP1. ***Conclusion:*** We show that both arms of miR-675 result in opposing effects on two downstream molecules MMP1 and MMP13. We propose that miR abundance may arise as response to direct target transcript levels and are thus dynamic to meet the requirements of the cellular environment.

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Introduction

MicroRNAs (miRs) are a large family of short (22-25 nt) non-coding RNAs that act as modulators of cellular mRNA stability and translation. miRs perform their regulatory function by base pairing via a short stretch of 7-8 nucleotides ("seed-sequence") with another mRNA. Once paired, a cellular machinery called the RNA Induced Silencing Complex (RISC) leads to mRNA degradation or translational block [1, 2].

The miR literature is heavily focused on identification of direct targets, i. e. these that can be determined as such by having a seed sequence match that would allow direct targeting of an mRNA by the studied miR. Due to redundancy of short sequence runs in the transcriptome there are many mRNAs within a cell that can be targeted by the same miR. Equally, a single mRNA can be targeted by many different miRs. Consequently, the final effect on the cell is not solely defined by the interaction between the miR and the mRNA target(s) but further on amplified by non-direct downstream effects that arise due to the primary effect between the miR and the direct target(s). This is of particular significance if the direct target is a transcription factor and its downregulation affecting many of its downstream dependent genes. Literature that investigates transcriptomic changes after miR overexpression confirms this observation, highlighting the importance of observing the total effect of a miR on a cell rather than focussing solely on direct targets [3-5].

Following transcription, the primary miR transcript is present as a hairpin structure, on which two different arms of the miR are encoded. In the early years of miR research it was thought that only one of these two arms will be active and the other one destined for degradation. The theory behind how the cell selects the future active arm was based on hydrogen bonding strength between the miR and the RISC, where the sequence with the higher stability would more likely be incorporated in the RISC, resulting in stabilization of this miR-arm [6]. In those days, the future active/inactive arms were termed miR/miR*. More recently it has however been shown that this observation does not hold true for many miRs, i. e. there are many examples where both arms of the same precursor miR can be detected at high or near equal levels and thus a different nomenclature, 5' and 3' arm with respect to orientation within the precursor molecule, was adopted [7, 8].

Activity of a miR requires associations with the RISC, thus detecting miR presence is not a satisfactory demonstration whether a given miR is active within the cell or not. This becomes of particular importance for miRs that are expressed at very low levels, where determination of presence is not a sufficient argument for potential activity.

The miR of the current study, miR-675, is expressed at low levels in human articular chondrocytes. However, previous research showed that miR-675 contributes positively to levels of type II collagen (COL2A1), the major collagen component in human articular cartilage [9], indicating an active role of this miR. COL2A1 is turned over by members of the Matrix-Metalloproteinase (MMP) family, in particular MMP1, MMP8 and MMP13 which are able to cleave and unwind the triple helical structure of the collagen molecule making it available for further degradation [10, 11]. These MMPs have important roles during clearance of collagen in development but also gained fame in context of joint and cartilage disease, especially within the field of arthritis [12-14].

In the present manuscript we determine the 3' arm of miR-675 as the active miR using AGO2 immunoprecipitation and show that it is approximately 10 times more abundant than the 5' arm in human articular chondrocytes. We demonstrate that miR-675-3' is able to indirectly downregulate MMP1 and MMP13 transcript and enzyme levels. We show that miR-675 arm abundance can be modulated, and highlight this at the example of myoblast differentiation, where levels of miR-675-5' are in close balance with miR-675-3'. We further on determine that the 5' arm has potent ability to upregulate MMP1 and MMP13 which contrasts with the action of the 3' arm. We conclude that for miR-675, arm abundance and activity are a dynamic process in a response to meet the cell's requirements for transcriptome regulation.

Materials and Methods

Human Articular Chondrocyte (HAC) isolation and culture

Normal healthy knee articular cartilage (age range 9-66 years; mean±SD of 27.2±21.9 years; 11 males, 4 females) was obtained from amputations due to sarcomas not involving the joint space as previously described [15]. Osteoarthritic cartilage samples (age range 45-81 years; mean±SD of 64.2±11.1 years; 12 males, 8 females) were obtained from patients undergoing total or unilateral knee replacement. HACs were isolated as described previously [16]. Briefly, HACs were digested from cartilage using collagenase A (Roche; catalog no. 10103578001) at 1.5 mg/gram of tissue over night at 37°C in 1xDMEM supplemented with 1x penicillin/streptomycin and fungizone. Following digestion, HACs were filtered through a 70-µm cell strainer and seeded in 10 cm cell culture dishes (passage 0). After having reached ~70% confluence, cells were split and seeded in 12- or 6-well dishes for experiments (passage 1). Cells at passage 1 and 2 were used in experiments.

For Fig. 2, 5*10E5 freshly isolated HACs after over-night digestion were spun down, washed once with DPBS and resuspended in Trizol. The allocation of donors for each experiment can be found as Table 1.

Table 1. List of donors used in each experiment

Non/OA	Gender	Age	Diagnosis	Type	Used in Figure #
OA	M	78	OA	Fi	2
OA	M	81	OA	Fi	2
OA	F	71	OA	Fi	2
OA	M	60	OA	Fi	2
OA	M	76	OA	Fi	2
OA	M	74	OA	Fi	2
OA	F	76	OA	Fi	2
OA	F	68	OA	Fi	2
OA	F	77	OA	Fi	2
OA	M	66	OA	Fi	2
OA	M	73	OA	Fi	2
OA	F	68	OA	Fi	2
OA	M	56	OA	P2	1
OA	F	45	OA	P2	1
OA	M	49	OA	P2	1
OA	M	45	OA	P2	3, 5, S1
OA	M	62	OA	P2	3, S1
OA	M	54	OA	P2	5, S1
OA	F	61	OA	P2	5, S1
OA	F	63	OA	P2	5, S1
Non-OA	M	35	Ewing's Sarcoma	Fi	2, S2
Non-OA	M	55	Myxofibrosarcoma	Fi, P2	2, 3
Non-OA	M	22	Soft tissue- and osteosarcoma	Fi	2, 3
Non-OA	M	13	Fibromatosis	Fi	2, 3
Non-OA	M	13	Osteosarcoma	Fi, P2	2
Non-OA	M	14	Osteosarcoma	Fi, P2	2, S2
Non-OA	M	9	Osteosarcoma	Fi	2
Non-OA	M	47	Myxoid liposarcoma	Fi	2
Non-OA	M	47	Chondrosarcoma	Fi	2
Non-OA	F	58	Chondrosarcoma	Fi	2, S2
Non-OA	M	66	Chondrosarcoma	Fi, P2	1, 2
Non-OA	F	68	Spindle Cell Sarcoma	P2	3, 5, S1
Non-OA	F	15	Osteosarcoma	P2	1
Non-OA	F	16	Ewing's Sarcoma	P2	5, S1

Transfection of miR mimics and inhibitors

Mimics and inhibitors were purchased from Life Technologies: mirVana™ mimics for miR-675-5' (4464066, MC12067), miR-675-3' (4464066, MC14423) let-7e-5' (4464084, MC12304). and mirVana™ inhibitors for miR-675-5' (4464084, MH12067) and miR-675-3' (4464084, MH14423),, Negative controls were 4464061 (mimic) and 4464076 (inhibitor) both with sequences proprietary to the manufacturer. HACs were gown in 6 well plates to 80% confluence, washed twice with warm DPBS and 2 mls serum-containing media was added to each well. Mimics were transfected at 15 nM concentration, inhibitors at 30 nM using Lipofectamine® 2000 (Thermo Fisher 11668027); complexes were formed in Opti-MEM™ (Thermo Fisher 31985062) for 15 minutes prior to dropwise addition to the cells. The next day, wells were washed, and media was replaced to serum free DMEM containing 1xITS (Thermo Fisher 41400045) and incubated for three days. Media was harvested on ice, debris was spun out for 10 minutes at 10000g at 4°C and cell culture supernatants were stored at -80°C until used. HACs were lysed in 0.6 ml Trizol (Thermo Fisher 15596018) per well.

Western blotting

Serum free supernatants were concentrated 15-fold with Amicon® Ultracel Centrifugation filters with a 10K MW cut-off (Sigma Z740171-24EA) and separated over a 4-20% Tris-Glycine gel (Thermo Fisher XP04205BOX). Proteins were transferred to a PVDF membrane and blotted by over-night incubation with antibodies against MMP1, MMP13 and Transferrin (all from abcam; ab52631, ab39012 and ab10208). Blots were developed with Clarity™ ECL reagent (Biorad #1705060).

Enzymatic activity assays

The SensoLyte® Enzymatic Activity assays for MMP1 (AS-71150) and MMP13 (AS-72019) were used to determine levels of both MMP1 and MMP13 in cell culture supernatants (SNs). SNs were concentrated 10-fold using Amicon® Filters (Sigma Z740171-24EA) and 100 µl of concentrated SNs were used in each well of the assay. Data was read with a FLUOstar Omega instrument (BMG Labtech) at Extinction 420/Emission 520 and relative fluorescence units were used to determine enzymatic activity.

Differentiation of Human Skeletal Myoblasts

Human Skeletal Myoblasts (Thermo Fisher A12555) were seeded into 48 well plates at 5*10E5 cells/well in low glucose DMEM (Thermo Fisher 11885084) supplemented with 2% Horse Serum (Thermo Fisher 16050130) and harvested after three and 72 hrs.

AGO2-RNA immunoprecipitation

Confluent HACs (one 10 cm dish per experiment) at passage 2 were washed twice with DPBS, scraped with ice cold DPBS into 15 ml conical tubes, spun down at 500g for 5 min and lysed in 0.9 ml of 1x lysis buffer (LB, Tris-Cl pH 7.4, NaCl, Igepal C630, 1 mM EDTA). Sepharose G Fast flow beads (Sigma P3296-5ML) were washed three times with LB, coated for one hour with 10 µg BSA in 1ml LB, washed again three times and coupled to 2 µg AGO2 antibody (Novus H00027161-A01) of IgG isotype control (Novus NBP1-97019) for 5 hrs, after which beads were washed again three times to remove unbound AGO2/IgG. The lysate was cleared by spinning at 10000g for 10 min at 4°C and the supernatant was equally distributed between two tubes containing beads coupled to isotype IgG control or beads coated with AGO2. After over-night rotation at 4°C, beads were washed five times with 1x LB and RNA extracted using Trizol (Thermo Fisher 15596026) reagent. RNA purification and reverse transcription was performed as described below.

RNA isolation, reverse transcription and qPCR

HACs were lysed in Trizol and RNA was purified using the Zymo RNA Clean and Concentrator kit (Zymo R1050). For reverse transcription of total cDNA, the VILO RT kit (Thermo Fisher 11766050) with 300-500 ng RNA was used according to the manufacturer's instruction. For reverse transcription of miRs, 50 ng total RNA was used with the TaqMan™ MicroRNA reverse transcription kit (Thermo Fisher 4366596) and RT primers for RNU24 (4427975, 001001), miR-675-5 (A25576, 478195_mir), miR-675-3 (A25576, 478196_mir), miR140-5' (A25576, 477909_mir), miR-140-3' (A25576, 477908_mir), and let-7e (4427975, 002406) all Thermo Fisher. QPCRs were performed on a Viia7 with SYBR™ green for total cDNA, (Thermo Fisher 4385612) or TaqMan for microRNA analysis (Thermo Fisher4304437)

technology. The efficiency of primer/probe combinations for both arms of miR-675 was evaluated using a copy number standard curve and determined to be 105% for miR-675-5' and 96.6% for miR-675-3'. Expression levels of miRs were normalized to RNU24 and those of MMP1 and MMP13 were normalized to RPLP0 and relative levels were evaluated using the ΔCt method. Following primers were used: RPLP0-FWD, CCATTGAAATCCTGAGTGATGTG; RPLP0-REV, CTTCGCTGGCTCCCACTTT; MMP13-FWD, AGACTTCCCAGGAATTGGTGA; MMP13-REV, ACGGTTACTCCAGATGCTGT; MMP1-FWD, CCAGGTATTGGAGGGGATGC; MMP1-REV, GTCCAAGAGAATGGCCGAGT, H19-FWD, ACCCACAACATGAAAGAAATGGT; H19-REV, GAGGGTTTTGTGTCCGGATTC; WWP2-FWD, CGTCAAGAACTCAGGCCACA WWP2-REV CAACGGAAGGTTCTTCGGGA .

Statistical Analysis

Based on normal distribution of the data, paired or non-paired two-tailed t-tests were used. Results are summarised as mean with standard deviation. Statistical significance was set at $p<0.05$. The primary and secondary outcome measures were mRNA and miR levels determined by qPCR and evaluated by the ΔCt method as well as protein levels, determined by western blot or enzymatic activity, measured in relative fluorescence units (RFU). The software package GraphPad Prism 7.4 was used for analysis. Human articular chondrocytes were derived from both healthy and osteoarthritic cartilage. The allocation of donors for each Figure is indicated in Table 1.

Results

miR-675-3' is the active arm in human articular chondrocytes and is overexpressed in OA chondrocytes

In order to establish the functional, active arm of miR-675 in HACs, we determined levels of both miR arms in the AGO2 complex, the functional component of the RNA induced silencing complex (RISC). As it can be seen in Fig. 1A, only the 3' arm displays significant enrichment in the AGO2 complex, but immunoprecipitated miRs indicate that the 5' arm is not present above background levels. In contrast, both arms of miR-140, one of the highest expressed miRs in human articular chondrocytes, are bound to AGO2, indicating functional activity for both arms of miR-140 (Fig. 1C). Notably, also H19, the host gene of miR-

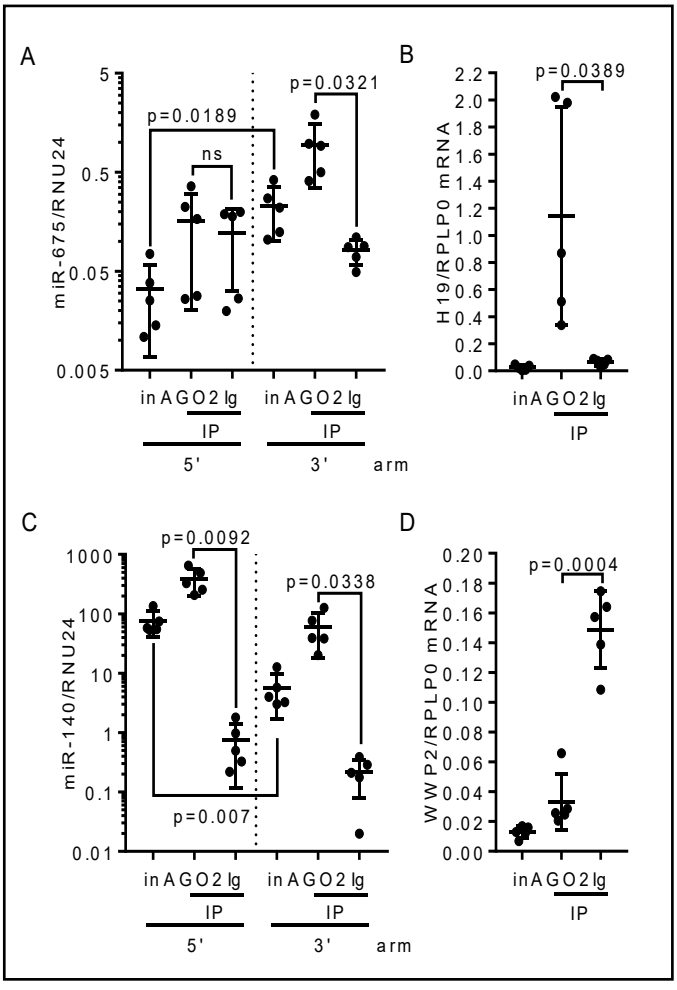

Fig. 1. miR-675-3' is the functional arm in adult human articular chondrocytes. A-D, RT-qPCR for miR-675-5' and 3' (A), miR-140-5' and 3' (C) and their respective host gene H19 (B) and WWP2 (D) in input fractions and after immunoprecipitation with AGO2 antibody. miR levels were determined by TaqMan technology and genes were analysed by SYBR Green. Statistical analysis was carried out with a paired, two-tailed t-test. In, input; AGO2, IP with AGO2; Ig, IP with IgG; IP, immunoprecipitation.

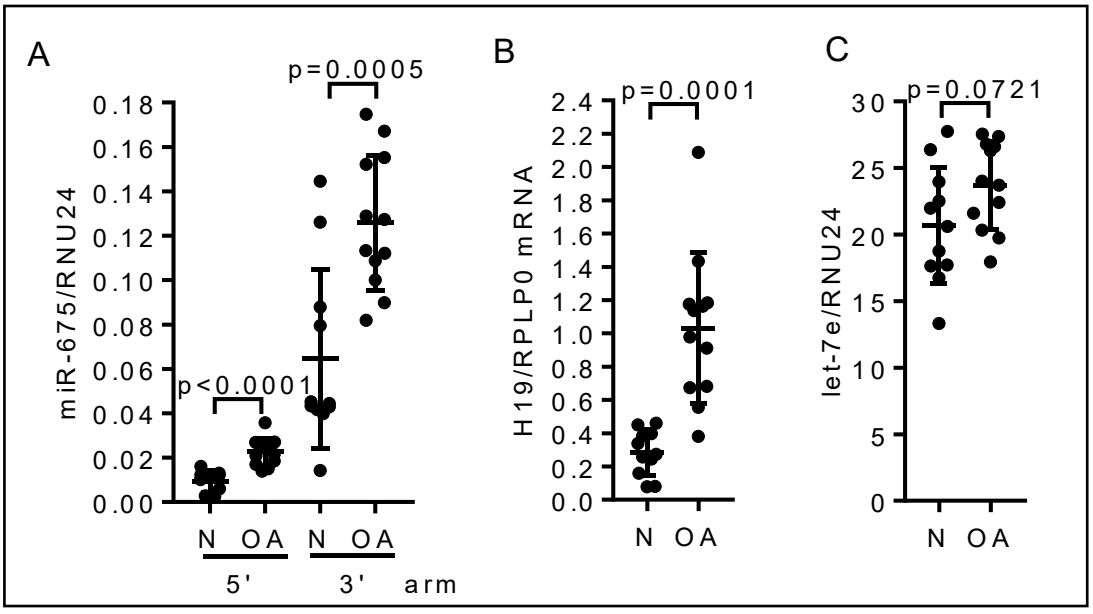

Fig. 2. Elevated levels of miR-675 in OA chondrocytes. A, levels of miR-675-5' and 3' (A) and their host gene H19 (B), as well as let-7e (C) in freshly isolated chondrocytes from healthy and OA affected cartilage. miR levels were measured by TaqMan assays, and H19 by SYBR Green RT-qPCR. Statistical analysis was carried out with a non-paired, two-tailed t-test. N, non-OA; OA, osteoarthritis.

675 is highly enriched in the AGO2 complex (Fig. 1B). Binding of WWP2, the host gene of miR-140 was however not found to be significant. Additionally, analysis of input fractions demonstrated that for miR-675, the 3' arm is approximately 10 times more abundant as compared to the 5' arm and for miR-140, the 5' arm is approximately 10 times above the level of the 3' arm. We next sought to establish whether the 3'-arm preference is also true in OA chondrocytes and compared levels of both arms of miR-675 in freshly isolated osteoarthritic (OA) and non-OA chondrocytes. We find increased expression of both the host gene, H19, as well as miR-675 in OA chondrocytes, with miR-675-3' being the predominantly detectable arm (Fig. 2A, B). There was no change in expression levels for let -7e, a ubiquitously expressed miR in both cell types (Fig. 2C). We conclude that in mature human articular chondrocytes, miR-675-3' constitutes the main active form.

miR-675-3' reduces levels of MMP1 and MMP13

Previous work established that overexpression of miR-675 results in increased levels of COL2A1, the major collagen component of articular cartilage (9). We thus focused our interest on a group of three collagen degrading enzymes, which are part of a large family of Matrix-metalloproteinases (MMPs), namely MMP1, MMP8 and MMP13 to determine whether miR-675 affects expression of one or more collagenases. Preliminary experiments determined that two of the three members of the collagenase family, MMP1 and MMP13, were expressed at significant levels in HACs and are well established in context of collagen turnover in healthy and diseased tissue. We thus continued investigating these two MMPs. Having established miR-675-3' as the active form, we overexpressed this arm using miR mimics and find transcriptional downregulation of both MMP1 and MMP13, with statistical significance for MMP1 and a tendency (although without statistical significance) for MMP13 (Fig. 3A and C). In order to determine whether this can be observed on the level of enzymatic activity, we performed MMP cleavage assays with two commercially available kits that have enhanced sensitivity for either MMP1 or MMP13. As it can be seen in Fig. 3B and D, reduction of enzymatic activity after miR-675-3' transfection can be observed for both enzymes; the effect on MMP1 is once again more dominant as compared to MMP13. Thus, miR-675-3' is able to reduce levels of both MMP1 and MMP13. We used inhibition of miR-675-3' with

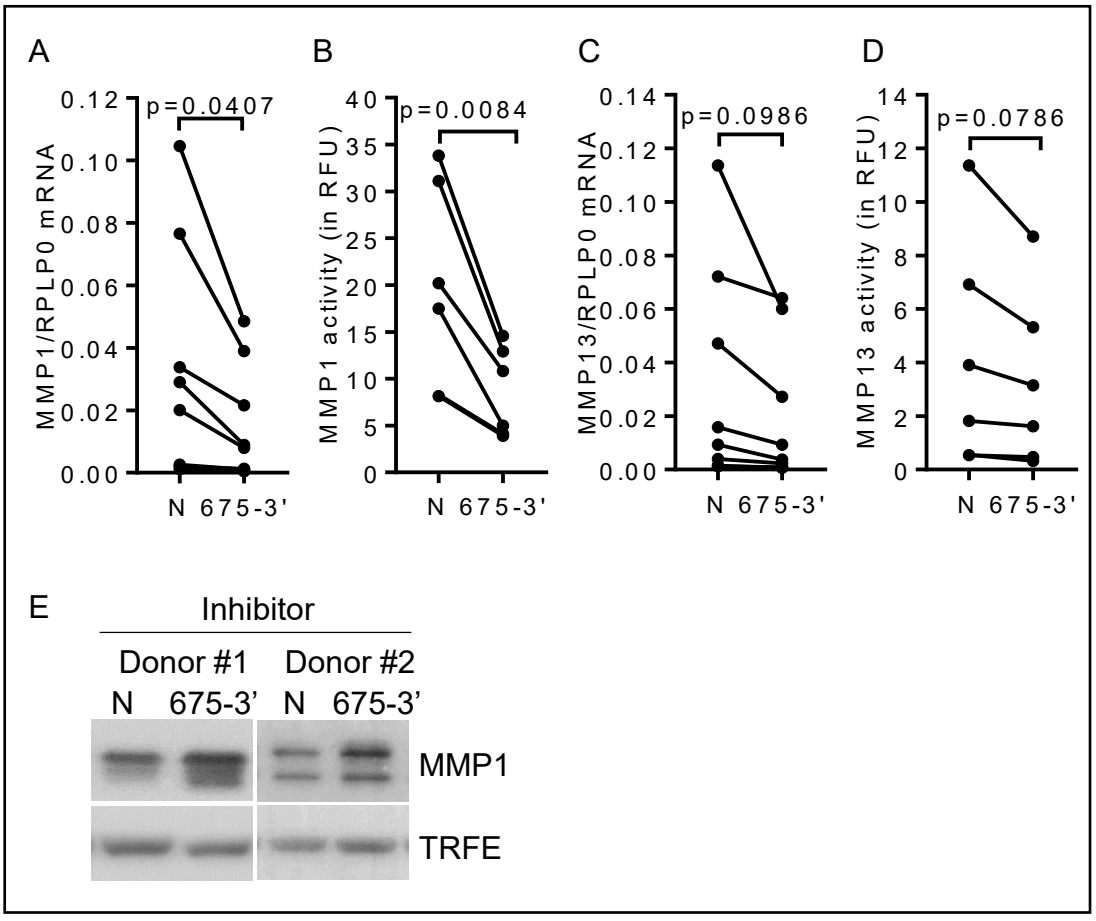

Fig. 3. miR-675-3' reduces levels of MMP1 and MMP13. A, C; RT-qPCR for MMP1 and MMP13 of RNA from human articular chondrocytes treated with negative control mimic or mimic for miR-675-3' measured three days post transfection. B, D, determination of enzymatic activity for MMP1 (B) and MMP13 (D) in cell culture supernatants of cells treated with negative control or miR-675-3' mimic after three days of transfection. E, western blot for MMP1 form cell culture supernatants on day three after treatment with negative control inhibitor or inhibitor against miR-675-3'. Statistical analysis was carried out with a paired, two-tailed t-test. N, negative control mimic/inhibitor; 675-3', mimic/inhibitor for miR-675-3'. Statistical significance was set at $p \leq 0.05$. TRFE, transferrin.

antisense inhibitors and determined that inhibition of natural levels of miR-675-3' results in slight but visible upregulation of MMP1 protein levels (Fig. 3E). Note that base levels of MMP13 were too low in these donors to be verified by western blotting.

High levels of both miR-675-5' and 3' are expressed during myoblast differentiation

The host gene of miR-675, H19, is a developmentally regulated gene whose expression is strictly downregulated in a majority of adult healthy tissues, with the exception of human skeletal muscle and articular chondrocytes that maintain high and medium levels of expression of H19. We were interested to determine relative levels of both arms of miR-675 in human adult skeletal muscle (aSkM) mRNA from two commercial suppliers and during myoblast differentiation to establish whether adult healthy tissue is normally associated with predominant miR-675-3' expression. We looked at the model of myoblast differentiation, during which myotubes are differentiated in low serum containing media to form into myotubes and determined levels and ratios of both arms of miR-675 by RT-qPCR 3 and 72 hrs after onset of differentiation. We find high expression of H19 and equally high expression of miR-675 at both time points of myoblast differentiation (Fig. 4A, B) and lower

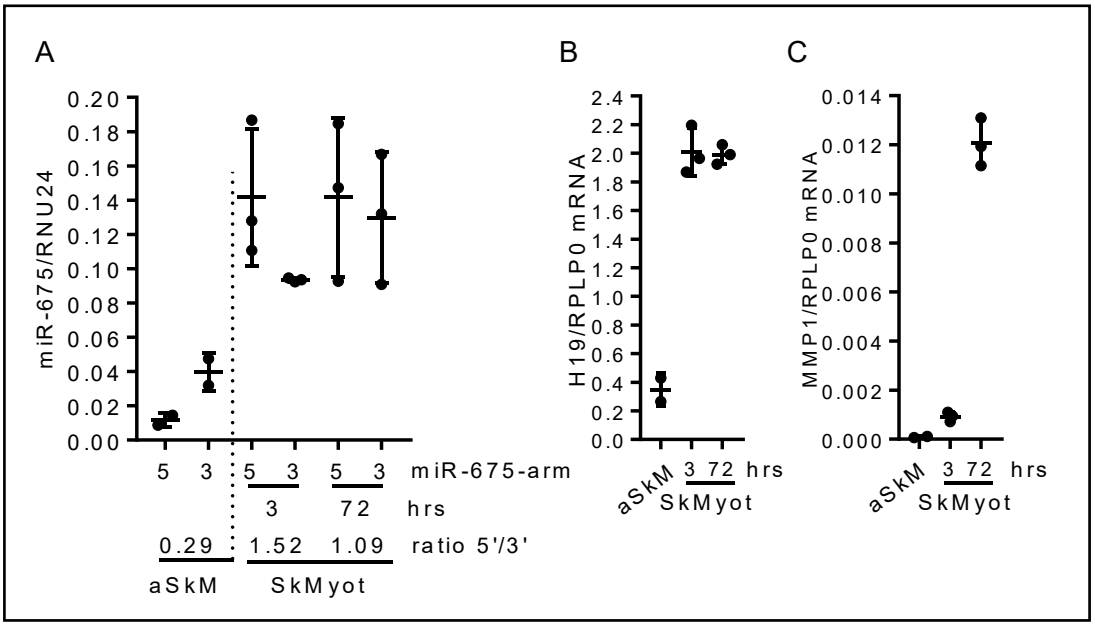

Fig. 4. High levels of both miR-675-5' and 3' are expressed during myoblast differentiation. TaqMan RT-qPCR for miR-675-5' and 3' (A) and SYBR RT-qPCR for H19 (B) and MMP1 (C) in two samples of aSkM tissue and three series of differentiation of skeletal myotubes. aSkM, adult skeletal muscle; SkMyot, skeletal myotubes. The two data points for aSkM originate from two RNA preparations from two different commercial suppliers.

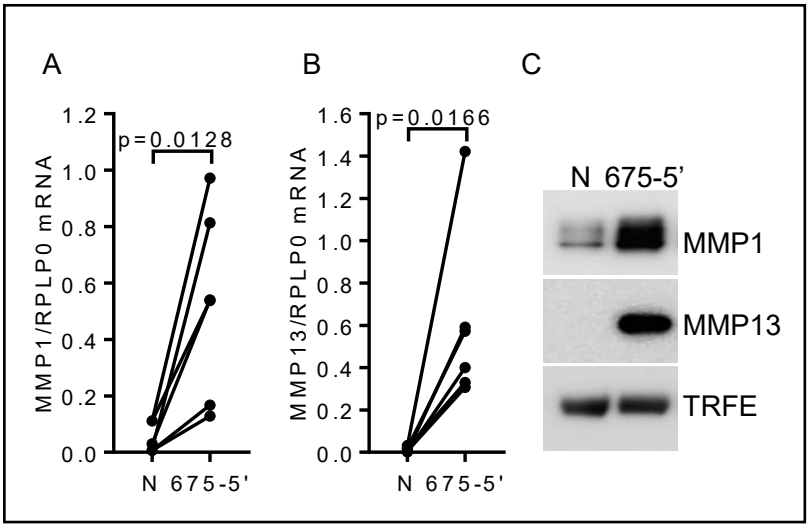

Fig. 5. miR-675-5' induces expression of MMP1 and MMP13. SYBR RT-qPCR for MMP1 and MMP13 on day three after overexpression of a negative control mimic or mimic for miR-675-5'. C, western blot of cell culture supernatants from samples used in A and B. Statistical analysis was carried out with a paired, two-tailed t-test. N, negative control; 675-5', mimic for miR-675-5'. TRFE, transferrin.

levels of both miR-675 and H19 in aSkM. Interestingly, in the myotube differentiation model, both arms of miR-675 are highly expressed in near similar ratios, indicating that both arms of miR-675 are functionally important. Notably, we also find an increase in expression of MMP1 during myoblast differentiation, indicating a role for this MMP during the process (Fig. 4C).

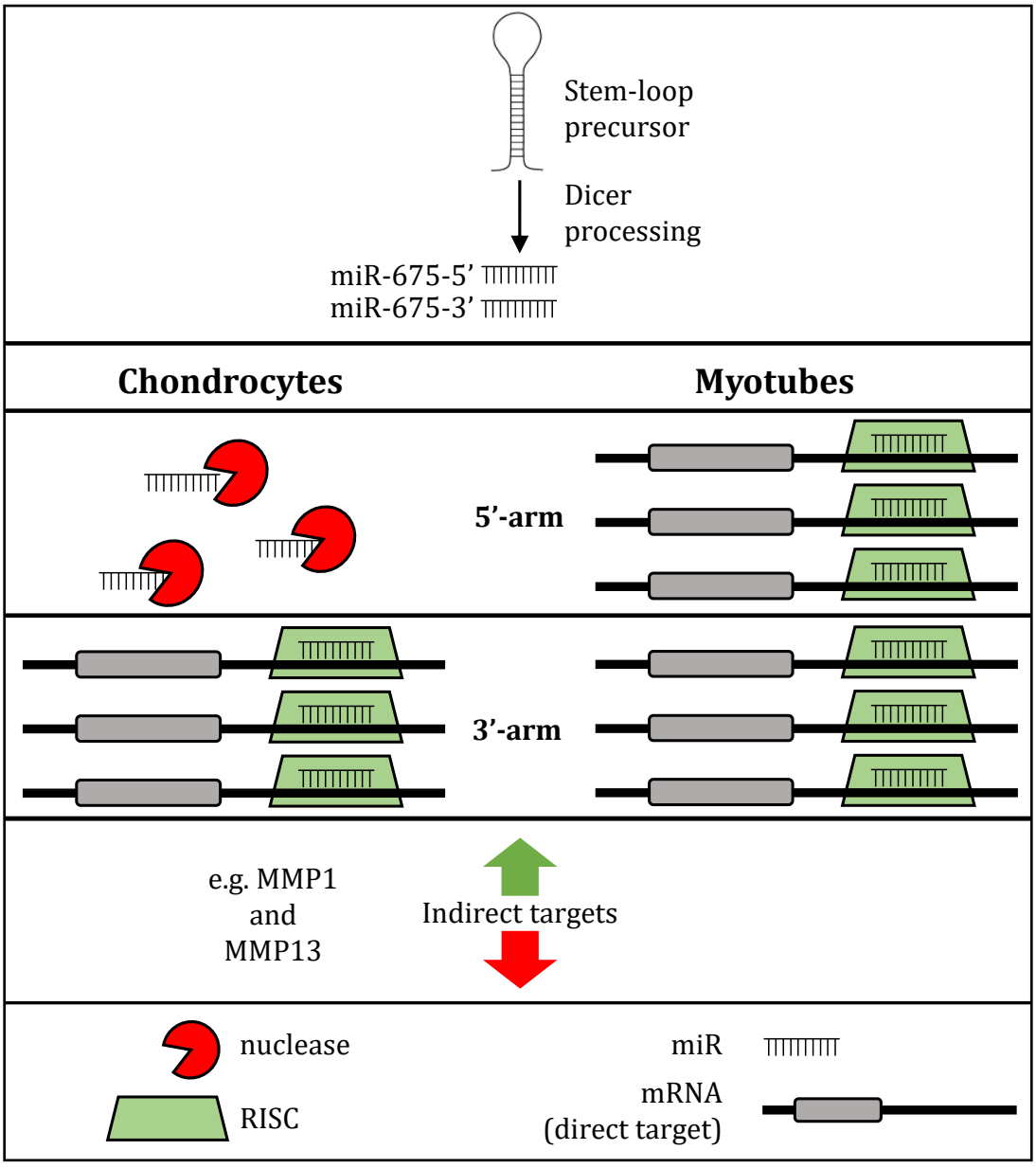

Fig. 6. miR-675 arm levels are determined by target availability. miR-675 is processed from the stem-loop precursor by Dicer, leading to two mature forms, miR-675-5' and miR-675-3'. In chondrocytes, the 5' arm is degraded by cellular nucleases due to low or no direct target availability. The 3' arm on the other hand, maintains steady state levels due to presence of direct targets. In myotubes, high levels of both miR-675 arms are present, possibly as a result of sufficient expression of direct targets. Indirect targets (e.g. MMP1 and MMP13 in chondrocytes) are affected downstream of this process resulting in elevated or decreased levels of mRNA and/or protein. RISC, RNA induced silencing complex.

miR-675-5' induces expression of MMP1 and MMP13

Given the observation of high levels of both arms of miR-675 during human myotube differentiation, we overexpressed miR-675-5' in human articular chondrocytes to determine whether – if overexpressed – there is a potential role for this arm of the miR. Surprisingly, we find an exact opposite effect on MMP1 and MMP13 as we had observed previously for the 3' arm, namely potent induction of both MMPs, visible on both mRNA and levels of secreted protein (Fig. 5A-C). In order to identify whether miR-675-5' and -3' also exert their

indirect functions on other members of the MMP family, we determined expression levels of ten more MMPs outside of the collagenase family representing members of the stromelysin, gelatinase and Membrane-bound MMP category. We found that four of them were expressed at significant levels in our system (Supplementary Table 1) and determined their response after overexpression of both arms of miR-675. However, we could only reconfirm reproducible up/downregulation of the initially investigated two MMPs, MMP1 and MMP13 (Supplementary Fig. 1 - for all supplemental material see www.cellphysiolbiochem.com). We also included a non-related miR control (let-7e) to exclude technically related changes. Summarizing, we conclude that the induction observed for miR-675-5' as well as reduction by miR-675-3' are specific and significant effects on MMP1 and MMP13 in human articular chondrocytes (Supplementary Fig. 2).

Discussion

In the present report we describe a mechanism of dual and opposing regulation of MMP1 and MMP13 by both arms of one miR, namely miR-675. We show that the 3' arm of miR-675 can reduce levels of MMP1 and MMP13 mRNA as well as of secreted protein. Overexpression of the 5' arm on the other hand results in potent transcriptional upregulation of both MMPs. This shows for the first time that both arms of a single miR can have opposing effects on transcriptional regulation and underlines the importance of not only miR expression but also tissue- and developmental specific arm-selection.

In context of arthritis and cartilage biology, Matrix-Metalloproteases (MMPs) are responsible for cartilage turnover and destruction by degrading one of the major cartilage components, collagen type 2 (COL2A1). Of the three collagenases MMP1, MMP8 and MMP13, the latter one is most potent in turning over COL2A1(10). MMP1 is found to be expressed at similar levels as MMP13 and also shows ability to cleave COL2A1, although less potently than MMP13 [17]. Under healthy conditions both MMP1 and MMP13 are expressed at low levels but are increased in expression in context of arthritic diseases. Whereas MMP1 is predominantly associated with rheumatoid arthritis, MMP13 has a demonstrated role in osteoarthritis and cartilage degradation [13, 18-22]. Equally, both MMPs display upregulation by inflammatory cytokines indicating that transcriptional regulation of MMP1 and MMP13 is linked in several pathways. Indeed, analysis of promotor regions of both genes identified sequence elements that indicate binding of transcription factors of the same families on both genes [23, 24]. Thus, it is feasible to find that the indirect effects by the two arms of miR-675 can be observed for both MMP1 and MMP13.

miR-675 is encoded within the first exon of another non-coding RNA gene, H19. This RNA is developmentally regulated with high expression in fetal tissues and very low expression in adult tissues except for skeletal muscle and articular cartilage that maintain high expression levels throughout adulthood [25, 26]. Interestingly, in spite of high H19 expression, levels of miR-675 vary between very low to medium during different stages of development in mouse embryo and extra-embryonic tissues. It was demonstrated that processing of miR-675 is inhibited by HuR, which associates with H19 in the RISC during development in mice [27]. However, knockdown of HuR by siRNA and analysis in cells genetically depleted of HuR only showed a moderate increase (2-4 fold) in levels of miR-675, indicating that HuR mediated trapping is only a minor factor regulating miR-675 abundance. Regulatory roles of both arms of miR-675 are focused on cell proliferation [28] and tumorigenesis/metastasis [29] in context of cancer cell lines and tissues and examples of direct targets identified and experimentally verified so far include GPR55 (non-small lung cell cancer) [30], RB (in colorectal cancer tissue)[31], CDH11 (fibroblasts and keratinocytes)[32], CDH13 (in glioma)[33] and FADD (in gastric cancer cell lines)[34]. miR-675 has also been found within exosomes of keratinocytes [35] and metastatic osteosarcoma [36] contributing to downregulation of CALN1 in the latter model. Little however is known about miR-675 in context of cartilage biology and OA. Dudek et al. [9] describe miR-675 as a promoting factor

for COL2A1 expression on both mRNA and protein levels in HACs isolated from healthy donors. Steck et al. [37] compared expression levels of miR-675 and H19 in OA and healthy tissue and find increased levels of both in OA, which is in agreement with the finding of the current manuscript. The authors also report reduction of transcript levels of COL2A1, H19 and miR-675 after treatment with inflammatory cytokines IL-1β and TNF-α.

The effect on MMP1 and MMP13 observed for miR-675-3' is indirect, since both MMPs lack a heptamer binding site that would allow for binding of the miR resulting in degradation of the mRNA. Given the modest but reproducible effect of downregulation, miR-675-3' may perform a threshold-guarding function, monitoring that MMP1 and MMP13 do not exceed a certain level. This could be achieved by directly targeting transcription factors that are normally involved in expression of these two MMPs. On the other hand, overexpression of the 5' arm leads to a very pronounced upregulation, thus miR-675-5' may directly target one or more repressors that would normally assure low levels of MMP1 and MMP13 expression. Although we have not set our focus on identification of direct targets of either arm of miR-675 (this has been carried out in depth by many other authors listed in the above paragraph), we would like to point out that indirect effects can be very dramatic and even outperform a direct interaction, since multiple direct interactions can amplify the end-result if these take place in a pathway that leads to regulation of an indirect target. This is certainly apparent in the upregulation of MMP1 and MMP13 by miR-675-5', leading to a median of 18.1 fold (MMP1) and 46.6 fold (MMP13) induction after overexpression of this miR. We thus argue that indirect effects reported are as valuable as direct interactions between a miR and an mRNA.

In the current study we show that during myotube differentiation levels of miR-675 are elevated and report a significant increase in the 5':3' arm ratio of miR-675 towards the 5' arm indicating that both arms are of functional importance in this model. Interestingly, in human adult skeletal muscle, levels of miR-675 dropped again and a preference of miR-675-3' could be observed. We agree that the N=2 for adult skeletal muscle only allows for careful speculation on this point but considering that also in mature human articular chondrocytes, miR-675-3' is the more abundantly detected arm, one could argue that the 5' arm is mainly required during the process of cellular differentiation, whereas miR-675-3' maintains functionality independent of cellular maturation. Given the observation of potent induction of MMP1 and MMP13 by miR-675-5', this makes sense since both MMPs have important function in tissue remodeling during development where higher levels of miR-675-5' are desirable to allow for support in elevated expression levels of MMP1 and MMP13. Equally, in adult human articular chondrocytes, where base expression of MMP1 and MMP13 is low, levels of miR-675-5' are very low and potentially non-functional since this arm is not detected in the RISC.

Interestingly, we noted a significant increase in expression levels of MMP1 during the differentiation process of myotubes. The involvement of MMP1 in myotube biology has been investigated by other groups and shown to be of benefit on several levels of myoblast differentiation and migration in context of wound healing [38, 39].

How the cell defines levels of both miR-arms is still incompletely understood. After the initial description of miR processing [1], nuclear export [40] and transcription by both RNA polymerases II and III [41, 42] little more research has been carried out to determine regulation of steady state levels of both arms of a given miR. Since both arms are encoded in the same miR precursor molecule, the difference in abundance of each arm of the same miR cannot be regulated on the transcriptional level. miR-arm preference has been mainly reported in context of disease where it was found that abundance of the 5' and 3' arms of certain miRs alter when comparing healthy to disease affected tissue [43-46] indicating that miR arm levels can respond to a change in the molecular environment. Furthermore, miR arm selection is developmentally regulated and cell type specific [47, 48] suggesting that modulation of miR-arm levels is a general observation due to alterations in cellular conditions. A mechanism of passive indirect regulation by providing increased stability of the active miR arm has been proposed recently [49]. In this model, the presence of target

mRNA molecules allows the miR to bind via its seed sequence match, followed by association with the RISC and this in turn prevents the miR from being degraded by cellular nucleases. The non-required arm of the miR (due to low or no levels of target mRNA) is degraded due to failure of being incorporated into a protective RISC. Thus, miR stability would be a consequence of target availability and a direct response to the change of the transcriptional landscape during development, in each cell type and in response to disease. A possible outline depicting this hypothesis for miR-675 is shown in Fig. 6. However, this model would make it necessary that each processed mature miR floats free in the cytoplasm for a certain amount of time finding their target mRNAs before being associated with the RISC – and only those arms that have found targets within that time would be protected from degradation. Indeed, there a no reports that show what happens between the Dicer processing step – which cleaves the hairpin from the precursor molecule generating the double stranded 5'/3' hybrid - and association of the mature miR with the RISC. This unknown interval between these two stages could be the determining time it takes for the miR to either be degraded (if no or low target levels can be found) or stabilized due to assembly with the target mRNAs and RISC.

miRs are branded as modulators of the transcriptomic landscape and thus it would make sense that their abundance arises due to the need for regulating an existing mRNA pool specific for each cell under different conditions. In the example of miR-675 in the current manuscript we show that levels of miR-675 as well as representation of arms are indeed dynamic and suggest that miR arm levels are determined by mRNA presence as a novel concept of miR stability and abundance.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Lee Y, Jeon K, Lee JT, Kim S, Kim VN: MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002;21:4663-4670.
2 Shukla GC, Singh J, Barik S: MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. Mol Cell Pharmacol 2011;3:83-92.
3 Shahab SW, Matyunina LV, Hill CG, Wang L, Mezencev R, Walker LD, McDonald JF: The effects of MicroRNA transfections on global patterns of gene expression in ovarian cancer cells are functionally coordinated. BMC Med Genomics 2012;5:33.
4 Matkovich SJ, Hu Y, Eschenbacher WH, Dorn LE, Dorn GW, 2nd: Direct and indirect involvement of microRNA-499 in clinical and experimental cardiomyopathy. Circ Res 2012;111:521-531.
5 Hill CG, Matyunina LV, Walker D, Benigno BB, McDonald JF: Transcriptional override: a regulatory network model of indirect responses to modulations in microRNA expression. BMC Syst Biol 2014;8:36.
6 Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD: Asymmetry in the assembly of the RNAi enzyme complex. Cell 2003;115:199-208.
7 Desvignes T, Batzel P, Berezikov E, Eilbeck K, Eppig JT, McAndrews MS, Singer A, Postlethwait JH: miRNA Nomenclature: A View Incorporating Genetic Origins, Biosynthetic Pathways, and Sequence Variants. Trends Genet 2015;31:613-626.

8 Budak H, Bulut R, Kantar M, Alptekin B: MicroRNA nomenclature and the need for a revised naming prescription. Brief Funct Genomics 2016;15:65-71.
9 Dudek KA, Lafont JE, Martinez-Sanchez A, Murphy CL: Type II collagen expression is regulated by tissue-specific miR-675 in human articular chondrocytes. J Biol Chem 2010;285:24381-24387.
10 Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534-1545.
11 Poole AR, Ionescu M, Fitzcharles MA, Billinghurst RC: The assessment of cartilage degradation in vivo: development of an immunoassay for the measurement in body fluids of type II collagen cleaved by collagenases. J Immunol Methods 2004;294:145-153.
12 Van Doren SR: Matrix metalloproteinase interactions with collagen and elastin. Matrix Biol 2015;44-46:224-231.
13 Burrage PS, Mix KS, Brinckerhoff CE: Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529-543.
14 van Meurs J, van Lent P, Holthuysen A, Lambrou D, Bayne E, Singer I, van den Berg W: Active matrix metalloproteinases are present in cartilage during immune complex-mediated arthritis: a pivotal role for stromelysin-1 in cartilage destruction. J Immunol 1999;163:5633-5639.
15 Lafont JE, Talma S, Murphy CL: Hypoxia-inducible factor 2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis Rheum 2007;56:3297-3306.
16 Seidl CI, Martinez-Sanchez A, Murphy CL: Derepression of MicroRNA-138 Contributes to Loss of the Human Articular Chondrocyte Phenotype. Arthritis Rheumatol 2016;68:398-409.
17 Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE: Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 1996;97:761-768.
18 Rowan AD, Litherland GJ, Hui W, Milner JM: Metalloproteases as potential therapeutic targets in arthritis treatment. Expert Opin Ther Targets 2008;12:1-18.
19 Maeda S, Sawai T, Uzuki M, Takahashi Y, Omoto H, Seki M, Sakurai M: Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis. Ann Rheum Dis 1995;54:970-975.
20 Green MJ, Gough AK, Devlin J, Smith J, Astin P, Taylor D, Emery P: Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. Rheumatology (Oxford) 2003;42:83-88.
21 Sato T, Konomi K, Yamasaki S, Aratani S, Tsuchimochi K, Yokouchi M, Masuko-Hongo K, Yagishita N, Nakamura H, Komiya S, Beppu M, Aoki H, Nishioka K, Nakajima T: Comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage. Arthritis Rheum 2006;54:808-817.
22 Xu L, Polur I, Lim C, Servais JM, Dobeck J, Li Y, Olsen BR: Early-onset osteoarthritis of mouse temporomandibular joint induced by partial discectomy. Osteoarthritis Cartilage 2009;17:917-922.
23 Chamberlain SH, Hemmer RM, Brinckerhoff CE: Novel phorbol ester response region in the collagenase promoter binds Fos and Jun. J Cell Biochem 1993;52:337-351.
24 White LA, Brinckerhoff CE: Two activator protein-1 elements in the matrix metalloproteinase-1 promoter have different effects on transcription and bind Jun D, c-Fos, and Fra-2. Matrix Biol 1995;14:715-725.
25 Lustig O, Ariel I, Ilan J, Lev-Lehman E, De-Groot N, Hochberg A: Expression of the imprinted gene H19 in the human fetus. Mol Reprod Dev 1994;38:239-246.
26 Goshen R, Rachmilewitz J, Schneider T, de-Groot N, Ariel I, Palti Z, Hochberg AA: The expression of the H-19 and IGF-2 genes during human embryogenesis and placental development. Mol Reprod Dev 1993;34:374-379.
27 Keniry A, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G, Reik W: The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol 2012;14:659-665.
28 Ma L, Tian X, Guo H, Zhang Z, Du C, Wang F, Xie X, Gao H, Zhuang Y, Kornmann M, Gao H, Yang Y: Long noncoding RNA H19 derived miR-675 regulates cell proliferation by down-regulating E2F-1 in human pancreatic ductal adenocarcinoma. J Cancer 2018;9:389-399.
29 Vennin C, Spruyt N, Dahmani F, Julien S, Bertucci F, Finetti P, Chassat T, Bourette RP, Le Bourhis X, Adriaenssens E: H19 non coding RNA-derived miR-675 enhances tumorigenesis and metastasis of breast cancer cells by downregulating c-Cbl and Cbl-b. Oncotarget 2015;6:29209-29223.

30 He D, Wang J, Zhang C, Shan B, Deng X, Li B, Zhou Y, Chen W, Hong J, Gao Y, Chen Z, Duan C: Down-regulation of miR-675-5p contributes to tumor progression and development by targeting pro-tumorigenic GPR55 in non-small cell lung cancer. Mol Cancer 2015;14:73.
31 Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, Kwok TT: Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. Carcinogenesis 2010;31:350-358.
32 Kim NH, Choi SH, Lee TR, Lee CH, Lee AY: Cadherin 11, a miR-675 target, induces N-cadherin expression and epithelial-mesenchymal transition in melasma. J Invest Dermatol 2014;134:2967-2976.
33 Shi Y, Wang Y, Luan W, Wang P, Tao T, Zhang J, Qian J, Liu N, You Y: Long non-coding RNA H19 promotes glioma cell invasion by deriving miR-675. PLoS One 2014;9:e86295.
34 Ge L, Zhang X, Hu S, Song Y, Kong J, Zhang B, Yang X: H19 suppresses the growth of hepatoblastoma cells by promoting their apoptosis via the signaling pathways of miR-675/FADD and miR-138/PTK2. J Cell Biochem 2018;120:5218-5231.
35 Kim NH, Choi SH, Kim CH, Lee CH, Lee TR, Lee AY: Reduced MiR-675 in exosome in H19 RNA-related melanogenesis via MITF as a direct target. J Invest Dermatol 2014;134:1075-1082.
36 Gong L, Bao Q, Hu C, Wang J, Zhou Q, Wei L, Tong L, Zhang W, Shen Y: Exosomal miR-675 from metastatic osteosarcoma promotes cell migration and invasion by targeting CALN1. Biochem Biophys Res Commun 2018;500:170-176.
37 Steck E, Boeuf S, Gabler J, Werth N, Schnatzer P, Diederichs S, Richter W: Regulation of H19 and its encoded microRNA-675 in osteoarthritis and under anabolic and catabolic in vitro conditions. J Mol Med (Berl) 2012;90:1185-1195.
38 Pan H, Vojnits K, Liu TT, Meng F, Yang L, Wang Y, Huard J, Cox CS, Lally KP, Li Y: MMP1 gene expression enhances myoblast migration and engraftment following implanting into mdx/SCID mice. Cell Adh Migr 2015;9:283-292.
39 Wang W, Pan H, Murray K, Jefferson BS, Li Y: Matrix metalloproteinase-1 promotes muscle cell migration and differentiation. Am J Pathol 2009;174:541-549.
40 Yi R, Qin Y, Macara IG, Cullen BR: Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 2003;17:3011-3016.
41 Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN: MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004;23:4051-4060.
42 Borchert GM, Lanier W, Davidson BL: RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 2006;13:1097-1101.
43 Li SC, Liao YL, Ho MR, Tsai KW, Lai CH, Lin WC: miRNA arm selection and isomiR distribution in gastric cancer. BMC Genomics 2012;13:S13.
44 Chang HT, Li SC, Ho MR, Pan HW, Ger LP, Hu LY, Yu SY, Li WH, Tsai KW: Comprehensive analysis of microRNAs in breast cancer. BMC Genomics 2012;13:S18.
45 Tsai KW, Leung CM, Lo YH, Chen TW, Chan WC, Yu SY, Tu YT, Lam HC, Li SC, Ger LP, Liu WS, Chang HT: Arm Selection Preference of MicroRNA-193a Varies in Breast Cancer. Sci Rep 2016;6:28176.
46 Li SC, Tsai KW, Pan HW, Jeng YM, Ho MR, Li WH: MicroRNA 3' end nucleotide modification patterns and arm selection preference in liver tissues. BMC Syst Biol 2012;6:S14.
47 Guo L, Yu J, Yu H, Zhao Y, Chen S, Xu C, Chen F: Evolutionary and expression analysis of miR-#-5p and miR-#-3p at the miRNAs/isomiRs levels. Biomed Res Int 2015;2015:168358.
48 Griffiths-Jones S, Hui JH, Marco A, Ronshaugen M: MicroRNA evolution by arm switching. EMBO Rep 2011;12:172-177.
49 Winter J, Diederichs S: Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. RNA Biol 2011;8:1149-1157.