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Mechanosensitive MiRs regulated by anabolic and catabolic loading of human cartilage

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SUMMARY

Objective: Elucidation of whether miRs are involved in mechanotransduction pathways by which cartilage is maintained or disturbed has a particular importance in our understanding of osteoarthritis (OA) pathophysiology. The aim was to investigate whether mechanical loading influences global miR-expression in human chondrocytes and to identify mechanosensitive miRs responding to beneficial and non-beneficial loading regimes as potential to obtain valuable diagnostic or therapeutic targets to advance OA-treatment.

Method: Mature tissue-engineered human cartilage was subjected to two distinct loading regimes either stimulating or suppressing proteoglycan-synthesis, before global miR microarray analysis. Promising candidate miRs were selected, re-evaluated by qRT-PCR and tested for expression in human healthy vs OA cartilage samples.

Results: After anabolic loading, miR microarray profiling revealed minor changes in miR-expression while catabolic stimulation produced a significant regulation of 80 miRs with a clear separation of control and compressed samples by hierarchical clustering. Cross-testing of selected miRs revealed that miR-221, miR-6872-3p, miR-6723-5p were upregulated by both loading conditions while others (miR-199b-5p, miR-1229-5p, miR-1275, miR-4459, miR-6891-5p, miR-7150) responded specifically after catabolic loading. Mechanosensitivity of miR-221 correlated with pERK1/2-activation induced by both loading conditions. The miR-response to loading was transient and a constitutive deregulation of mechano-miRs in OA vs healthy articular cartilage was not observed.

Conclusions: MiRs with broader vs narrower mechanosensitivity were discovered and the first group of mechanosensitive miRs characteristic for non-beneficial loading was defined that may shape the proteome differentially when cartilage tissue is disturbed. The findings prompt future investigations into miR-relevance for mechano-responsive pathways and the corresponding miR-target molecules.

Introduction

Articular cartilage is subjected to loading cycles and mechanical loading plays an important role in regulating development and maintenance of the tissue. One of the main factors regulating the metabolic activity of chondrocytes is dynamic compressive loading which can support extracellular matrix (ECM)-synthesis[1–3] or, under permanent improper loading, can inhibit matrix production, disrupt the ECM and lead to osteoarthritis (OA).[4,5] Early phases of OA are characterized by an activation of cells[6,7] and a stimulation of matrix-synthesis[8] whereas during later phases the degradation of matrix molecules prevails.[9] Although OA can be initiated by multiple factors, mechanical overloading is still the key feature of OA pathogenesis. The mechanisms by which different mechanical stimuli translate into a biological chondrocyte response are, however, still insufficiently understood. Accumulating evidence

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suggests that beyond transcription factors and signaling molecules, post-translational mechanisms such as those involving microRNAs (miRs), may be important in shaping the mechano-response. Thus, elucidation of whether and how miRs are involved in the mechanotransduction pathways by which cartilage is maintained or disturbed is highly relevant to our understanding of OA pathophysiology.

MiRs are post-transcriptional regulators of gene expression which may quickly adjust the cellular transcriptome in a stimulus-dependent manner. They are a class of non-coding RNA molecules that bind to mRNA and carry out epigenetic silencing of gene expression by triggering repression or degradation of the target-mRNA. Bioinformatic studies predict that miRs regulate more than 60% of protein-coding genes.[10] Each miR regulates multiple mRNA-targets and a cohort of cartilage-expressed miRs fine-tunes the chondrocyte proteome cooperatively. Several miRs were associated with the regulation of chondrocyte development,[11–14] joint homeostasis[15–17] and OA pathophysiology[18–22] and outside the joint, several miRs were linked with mechanosensitivity. MiRs have been implicated in mechanotransduction in human airway smooth muscle and epithelial cells[23,24] and in responses to shear and stretch forces in cardiovascular biology.[25] Surprisingly, despite the importance of load-bearing for maintenance and destruction of articular cartilage, little is known about mechanosensitive miRs and their potential role in cartilage function and pathology.

MiR-365 was the first cartilage-related mechanosensitive miR, described in chicken sternum chondrocytes cultured in collagen scaffolds[26] and was suggested to modulate cell proliferation and hypertrophic marker expression, which are regulated during OA development.[27,28] MiR-221/-222 were suggested to be mechanosensitive in bovine articular cartilage tissue since their expression was increased in the weight-bearing anterior compared to the posterior non–weight-bearing medial femur condyle[29] but their direct involvement in active regulation of mechanotransduction remains unclear. Single miRs like miR-27a, miR-140 and miR-146a were significantly regulated in human chondrocyte monolayer cultures exposed to hydrostatic pressure, but the absence of a cartilage ECM is a clear limitation of that study.[30] We recently performed a global transcriptome analysis of mature tissue-engineered cartilage exposed to a 3 h dynamic loading episode and demonstrated that chondrocytes responded by a significant stimulation of GAG-synthesis, an accumulation of SOX9 as well as stimulation of TGF-β-, BMP-, ERK1/2-, calcium- and WNT-signaling response genes indicating broad cell activation and an anabolic cell response.[31,32] However, the miR expression profile in response to loading was not assessed.

In summary, there is insufficient knowledge on a potential role of miRs in the mechano-response of chondrocytes, specifically in regard to the differential regulation of miRs with beneficial vs non-beneficial loading regimes and a link to expression levels in normal vs OA cartilage. Analyzing the mechanosensitive miR repertoire in human chondrocytes is thus a crucial step forward in understanding basic mechanisms of cartilage function and may help to identify novel diagnostic targets for OA development or for new therapies to improve quality of life for patients. Therefore, the aims of this study were to identify mechanosensitive miRs in tissue-engineered human cartilage regulated by cyclic unconfined compression, determine whether they are sensitive to beneficial and non-beneficial loading regimes and whether they are differentially expressed between OA cartilage and normal cartilage tissue, shaping the proteome differentially depending on a disease status. We hypothesized that a physiological loading episode representative of normal cartilage function may involve regulation of a different set of mechanosensitive miRs than a non-beneficial loading regime and postulated that, given mechanical overuse degenerated the cartilage, at least part of the “non-beneficial” miR pattern may be reproduced in late OA cartilage samples when compared with healthy cartilage tissue.

Methods

Tissue samples, preparation of engineered cartilage and dynamic compression

OA cartilage samples were taken from 21 patients and healthy tissue from 20 different subjects overall 41 distinct donors (Suppl. Table 1). One cartilage-chip of about 100 mg per donor was flash-frozen in liquid nitrogen and stored until use. Human articular chondrocytes (HACs) were isolated from cartilage of OA patients as described.[32] All donors provided informed consent. The study was approved by the local ethics committee (Medical Faculty of Heidelberg). The procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1,975, as revised in 2,000.

5×10^5 HACs were seeded in a collagen carrier, attached to the bone-replacement material β-tricalciumphosphat (β-TCP) and pre-cultivated under chondrogenic conditions containing 10 ng/ml TGF-β1 before exposed to mechanical stimulation.[33] Protocol A was designed in a previous study.[31] Briefly, day 35 constructs were stimulated by dynamic compression with an amplitude of 25% super-imposed on 10% static-offset with a frequency of 1 Hz in nine intervals of 10 min loading followed by 10 min of break over 3 h. For protocol B, constructs were pre-cultured for 21 days, cyclic unconfined compression was 25% super-imposed on 10% static-offset with a frequency of 0.5 Hz. In protocol B, the 3 h loading regime was reiterated 4 times within 24 h with 3 h of rest in between. 10% static-offset was applied during break and rest intervals. Controls were kept in the same device without loading.

Metabolic labeling

GAG-synthesis was measured after the end of the last loading interval as described previously.[32] Cartilage was detached from β-TCP, placed on a nylon mesh in a 48-well plate and labeled by 4 μCi $^{35}SO_4$ (Hartmann Analytic) in 500 μl chondrogenic medium for 24h. After washing 5 times in 1 mM Na_2SO_4 in PBS for 20 min while shaking, constructs were digested with 0.5 mg/ml proteinase-K at 60°C at 800 rpm overnight. Incorporated label of whole lysates was quantified by β-scintillation counting (1,414 Winspectral program) and baselined against the DNA-content.

Analysis of re-differentiation, protein and VLRH analysis

Procedures are described in detail in the supplemental information.

Constructs were fixed in Bouin's solution and matrix deposition was assessed in 5 μm paraffin sections via histological safranin O staining and immunostaining for collagen type II as described.[31] Total GAG deposition was quantified in proteinase-K digests with a DMMB assay[34] and referred to DNA-content by Quant iT PicoGreen.

Protein levels of pERK1/2 and ERK1/2 were detected via Western blotting in cell lysates as described before,[31] β-actin was used as a reference.

Unconfined indentation testing was performed using the very-low-rubber hardness (VLRH)-test method at DIN ISO27588 (Digitest II, Bareiss) as described previously.[33] Sample hardness was calculated from the difference in penetration depths that a ball

intender covers between an initial low contact force held for 5s and a high total force held for 30s.

Total RNA isolation, mRNA and miR expression analysis

Total RNA of cartilage tissue samples was isolated as described.[35] Briefly, 100 mg of cartilage was pulverized and RNA was isolated using a phenol/guanidine isothiocyanate extraction. For precipitation 1/3 volume of 1.2M sodium chloride and 0.8M of sodium acetate and 2/3 volume of 100% isopropanol was added. Precipitated RNA was washed, eluted and further purified using miRNeasy Mini Kit. Total RNA from constructs was isolated from mechanically minced samples by phenol/guanidine isothiocyanate extraction.

For mRNA analysis by qRT-PCR, total RNA was additionally purified using Zymoclean™ Gel DNA Recovery Kit, reverse transcribed, and gene expression was assessed relative to expression of the reference genes CPSF6 and HNRPH1 via the ΔCt method (Suppl. Table 2).

For miR expression analysis, total RNA was reverse transcribed and amplified using the TaqMan® MicroRNA method from Life Technologies. snRNU6 (U6) was used for normalizing miR detection signals.

MiR microarray analysis

Total RNA was subjected to miR expression analysis using Agilent Human microRNA Microarray v21.0 (2549 miRs; Agilent Technologies) where expression levels of 2549 miRs by 5893 loci were determined. Labeling of total RNA, array hybridization and microarray scanning were performed at the Genomics and Proteomics Core Facility at the German Cancer Research Center, Heidelberg. Fluorescence signals from individual beads were removed when the absolute difference to the median was >2.5-fold and fluorescence-intensity values were normalized applying quantile normalization.[36]

Statistical analysis

Microarray data were analysed by unpaired Significance Analysis of Microarrays (SAM). Following this approach quantile normalized and log2-transformed data of individual probes were compared to identify miRs differentially expressed between controls and compressed samples.[37] For multiple testing in SAM analysis correction with the median false discovery rate (FDR) with FDR set <0.05 was applied. Each engineered cartilage construct was considered as independent biological sample. For all other experiments values for corresponding unloaded control samples were set as one and comparison of compressed vs non-compressed groups were calculated using the non-parametric Mann–Whitney U test with $P < 0.05$ considered significant. Data analysis was performed using MultiExperiment Viewer 4.9.0 (TM4 Microarray-Software-Suite)[38] and SPSS 22.0.

Results

Selection of an anabolic and catabolic loading protocol

To test our hypothesis that mechanosensitive miRs are involved in the response of cartilage to dynamic loading and differ in expression dependent on an anabolic vs catabolic response, mature tissue-engineered cartilage was mechanically challenged by two different loading protocols. Protocol A was designed in a previous study to imitate 3 h of normal walking in 10 min intervals and caused a broad cell activation, stimulation of proteoglycan synthesis and an anabolic cell response.[31,32] It was applied at day 35 of pre-culture and consisted of a single 3 h loading episode in which 10 min intervals of cyclic loading were followed by 10 min intervals of pause [Fig. 1(A)]. Loading protocol B, designed to inhibit proteoglycan synthesis, was established by systematic variation of several parameters in a pilot study. Consistent results were obtained at day 21 of pre-culture when a 3 h loading episode with parameters depicted in Fig. 1(B) was repeated 4 times over 24 h with 3 h of break in between. Collagen type II and proteoglycans were evenly distributed throughout the cartilage according to histology after both selected pre-culture periods (Suppl. Fig. 1(A) and (B)). Using dual layered constructs we attempted to recreate the mechanical environment of the osteochondral unit and indeed the hardness of constructs tested via VLRH on day 21 reached 82% of values of native human articular cartilage plugs and 93% after a pre-culture time of 35 days. The model can, thus, be considered representative for native cartilage. Quantification of GAG-synthesis by radiolabel incorporation over 24 h confirmed a significant stimulation of GAG-production after application of protocol A (1.59-fold, $P = 0.010$, Fig. 1(C) and (a) significant downregulation after protocol B (-1.48-fold, $P < 0.0001$, Fig. 1(C)). While SOX9 mRNA was significantly upregulated by protocol A (3.09-fold, $P = 0.008$; Fig. 1(D)), SOX9 (-1.44-fold, $P = 0.010$), ACAN (-1.29-fold, $P = 0.001$) and COL2A1 mRNA (-1.69-fold, $P = 0.001$) were significantly downregulated by protocol B. Furthermore, with protocol B MMP3 expression raised by 20% and TIMP2 and four expression dropped by 33% in line with a detrimental loading response (data not shown). Exposure to protocol A did not significantly alter the GAG/DNA-content of samples within 24 h (Suppl. Fig. 1(C)) while GAG/DNA was significantly reduced by 19% ($P < 0.0001$) at the end of protocol B. Overall, an anabolic and a catabolic loading response was reproducibly induced in the tissue-engineered cartilage and the selected loading regimes were used for characterization of the miR-response.

MiR-profiling in response to anabolic stimulation

When global miR expression was determined by microarray analysis after exposure to protocol A in comparison to unloaded controls ($n = 4$ per group, four donors), no separation of loaded vs control samples was obtained after clustering of miR expression data. According to a scatter plot of SAM with median FDR <0.05 [Fig. 2(A)], only seven miRs were significantly upregulated in compressed vs non-compressed samples with only one being modulated more than twofold (Table I). Even when only data from the significantly regulated loci were used for clustering, groups were not separated, indicating little consistent miR regulation [Fig. 2(B)]. When three miRs with the highest differential expression were re-evaluated by qRT-PCR in partly independent samples ($n = 7$, seven donors) the significant upregulation of miR-6872-3p (1.55-fold, $P = 0.001$) and miR-6723-5p (1.46-fold, $P = 0.017$) was confirmed [Fig. 2(C)]. Levels of both miRs had returned to baseline by 4 h after the end of loading (data not shown). The weak regulation of very few miRs under physiologic loading conditions demonstrated that miR regulation was no dominant mechanism for the physiologic mechano-response after the chosen 3 h loading episode.

MiR-profiling in response to catabolic stimulation

When global miR expression analysis was performed for samples subjected to protocol B vs non-loaded controls ($n = 4$ per group; four donors) in independent experiments, hierarchical clustering of global miR data provided a clear separation of controls and compressed samples into two groups [Fig. 3(A)]. Unpaired SAM

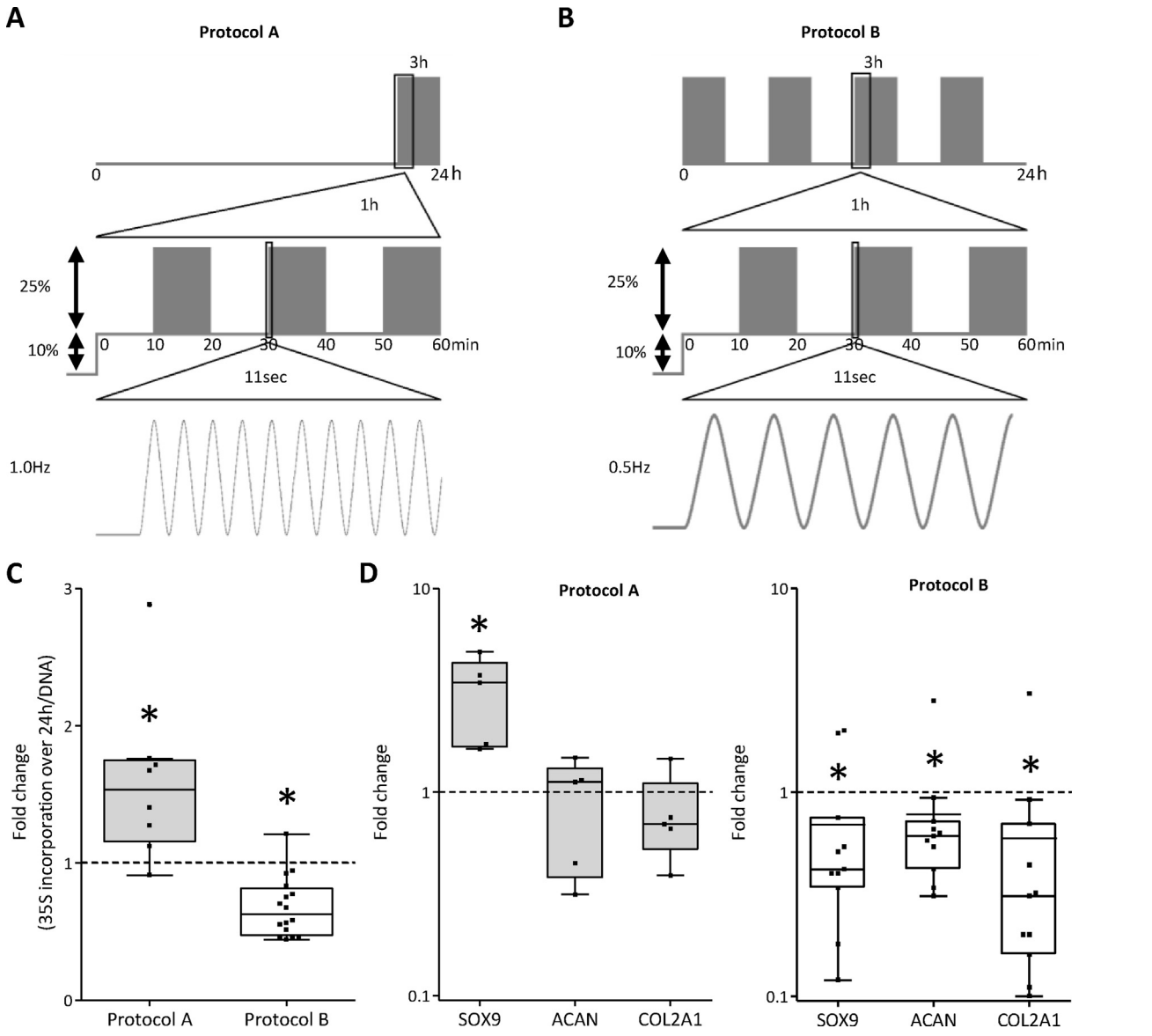

Fig. 1. Loading parameters and influence of dynamic loading on proteoglycan synthesis and gene expression. Constructs seeded with 5×10^5 human articular chondrocytes were pre-cultivated under chondrogenic conditions before subjected to dynamic unconfined compression. A: Protocol A applied at day 35 consisted of a single 3 h loading episode with an amplitude of 25% superimposed on 10% static-offset. During the 3 h loading episode nine intervals of 10min loading were separated by 10min of break where static-offset was applied. B: Protocol B applied at day 21 consisted of four 3h loading episodes within 24 h. Between each loading episode 3 h break was applied where constructs were exposed to 10% static-offset. C: Alterations in 35-S-sulfate incorporation into constructs for 24h following termination of loading to measure GAG-synthesis (A: $n = 8$ (5 donors); B: $n = 16$ (11 donors)). Values were normalized to DNA-content and non-loaded samples were set as 1 (dashed line). D: Gene expression analysis of constructs subjected to loading protocol A or B vs non-loaded controls. Total RNA was isolated from control and compressed samples (A: $n = 5$, four donors; B: $n = 11$, 11 donors) and mRNA levels were determined by quantitative polymerase chain reaction (qRT-PCR). Data were normalized to references genes (HNRPH1 and CPSF6) and non-loaded samples were set as 1 (dashed line). Data are shown as box plots with each box representing the interquartile range (IQR) extending between the 25th and 75th percentiles and lines inside the boxes represent the median and ■ represent single values. Whiskers extend to a maximum of 1.5 IQR. Mann–Whitney U test, * $P < 0.05$.

analysis with median FDR <0.05 [Fig. 3(B)] disclosed 80 significantly regulated miRs, 60 of which were downregulated and 20 of which were upregulated. Hierarchical clustering and heat map visualization of the significantly regulated miRs illustrated the significant differences between groups [Fig. 3(C)]. The top 10 significantly upregulated miRs increased over twofold while only one of the top 10 downregulated miRs decreased over twofold (Table II). The most strongly regulated miRs (bold in Table II) were re-evaluated via qRT-PCR in partly independent samples. Regulation of 5/5 tested upregulated miRs was confirmed [Fig. 4(A)] but a change in only 1/6 downregulated miRs was detectable [Fig. 4(B)]. Since miR-365 and miR-221/-222 were proposed to be mechanosensitive, their expression levels were also determined. MiR-365, being among the significantly downregulated miRs in microarray analysis (1.32-fold), showed no downregulation by qRT-PCR [Fig. 4(C)] but a significant upregulation of miR-221 (1.82-fold, $P < 0.0001$) and miR-222 (1.37-fold, $P < 0.0001$) was detected [Fig. 4(C)]. In conclusion, dynamic loading that leads to inhibition of proteoglycan synthesis and a catabolic cell response significantly influenced miR expression in chondrocytes, indicating that regulation of a set of mechanosensitive miRs may contribute to the mechano-response.

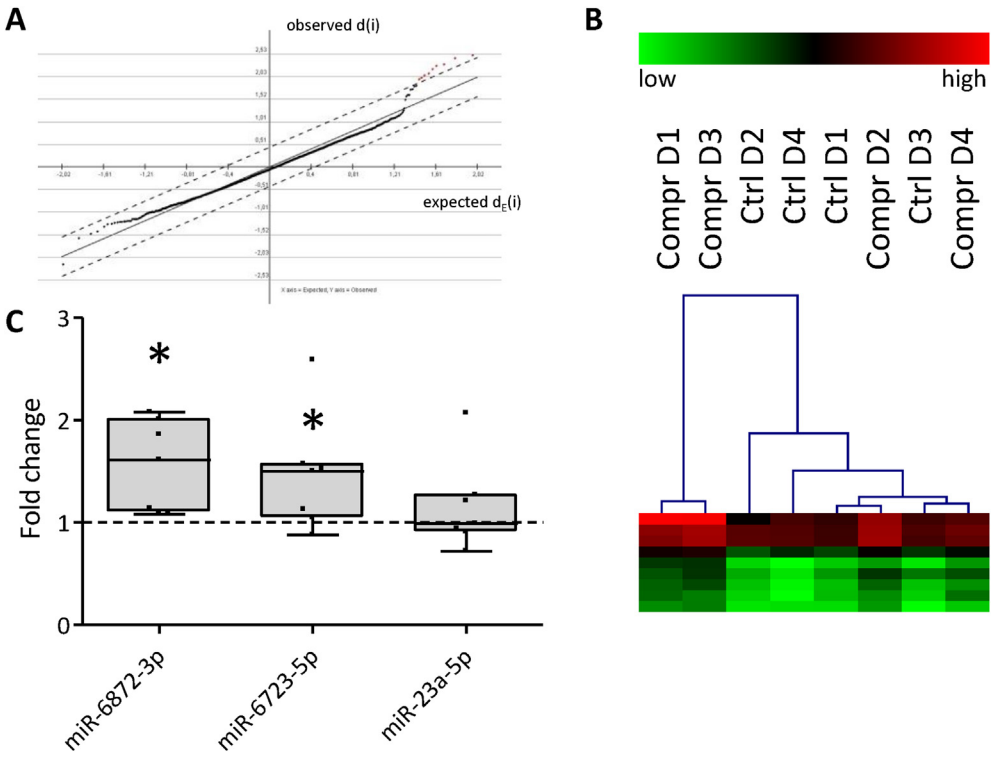

Fig. 2. Global miR expression in response to loading protocol A. Total RNA from eight samples ($n = 4$ per group; four donors) was subjected to global miR microarray analysis. A: Microarray results depicted as scatter plot following data processing by Significance Analysis of Microarrays (SAM). The observed relative difference d(i) was plotted against the expected relative difference dE(i) for control and compressed groups. Dashed lines define the difference between d(i) and dE(i) beyond which miRs are considered significant at a median false discovery rate (FDR) < 0.05. In red: probes significantly higher for compressed vs control group, green probes significantly lower in compressed vs control group. B: Hierarchical clustering and heat map visualization of significantly regulated probes ($n = 9$, seven miRs). Normalized and log-transformed expression data were color-coded by their level of expression. Green: low expression. Red: high expression. C: Expression levels of selected miRs according to qRT-PCR in samples used for microarray analysis and independently generated samples. Values were normalized to snRNU6 (%U6) and non-loaded samples were set as 1 (dashed line). Data are shown as box plots as indicated in Fig. 1. Mann–Whitney U test, * $P < 0.05$, $n = 7$, seven donors.

Table I
Significantly regulated miRs between control and compressed samples according to miR microarray analysis from protocol A

microRNA	Ctrl	+/− sd	Compr	+/− sd	Fold
hsa-miR-6872-3p	**118.33**	**+/− 15.18**	**262.26**	**+/− 91.23**	**2.22**
hsa-miR-6723-5p	**138.07**	**+/− 7.52**	**197.42**	**+/− 21.83**	**1.43**
hsa-miR-23a-5p	**51.74**	**+/− 3.16**	**68.44**	**+/− 4.70**	**1.32**
hsa-miR-513a-5p	57.65	+/− 6.06	75.46	+/− 2.98	1.31
hsa-miR-100-3p	50.38	+/− 3.52	65.19	+/− 2.58	1.29
hsa-miR-4734	77.07	+/− 3.74	97.88	+/− 7.31	1.27
hsa-miR-4793-3p	47.57	+/− 2.58	59.45	+/− 4.49	1.25

$n = 4$ per group; bold, miRs re-analysed by qRT-PCR.

Cross-testing of mechanosensitive miRs

With the stringent criteria applied for array data analysis, no overlap of candidate mechanosensitive miRs was recognized after loading with protocol A or B. To challenge the protocol-dependent regulation of the PCR-confirmed miRs, cross-testing was performed. Interestingly, miR-6872-3p and miR-6723-5p identified with protocol A were also significantly upregulated after protocol B, with similar amplitude (2.34-fold, $P < 0.0001$ and 1.77-fold, $P = 0.005$; Fig. 5(A)). In contrast, six miRs (miR-7150, miR-1275, miR-1229-5p, miR-6891-5p, miR-4459 and miR-199b-5p) identified with protocol B were only regulated by the catabolic loading regime. Interestingly, miR-221 was elevated by both loading protocols suggesting a broad mechanosensitivity. In order to check whether miRs recognized with protocol B, lasting 24 h, simply needed longer for upregulation than the 3 h applied with protocol A, these miRs were retested at 4, 8, or 21 h after the end of protocol A (corresponding to 7, 11 or 24 h after start of loading) but no delayed upregulation was observed (data not shown). In conclusion, a group of mechanosensitve miRs reacted specifically to catabolic loading, while three changed in both conditions, demonstrating that miRs with a broader vs narrower mechanosensitivity can be defined.

In vascular smooth muscle cells[39] and skeletal muscle cells[40] miR-221 expression was under the control of the MAPK-pathway and of ERK1/2 signaling. Mechano-responses usually involve ERK1/2 activation and Western blotting demonstrated that indeed pERK1/2 levels increased after loading with protocol A and B [Fig. 5(B)]. In order to further test whether miR-221 upregulation may correlate with pERK1/2 activation, loading with protocol A was performed on day 3 ($n = 4$ donors) when no pERK activation was observed, possibly due to the immaturity of the samples [Fig. 5(C)]. Indeed, no miR-221 upregulation was observed in the absence of pERK1/2 activation [Fig. 5(D)]. Furthermore, when pERK signaling was inhibited by UO126 2 h before loading and during the 24 h of loading by protocol B, UO126 reduced the phosphorylation of ERK in response to loading as evident from Western blotting (Supplemental Fig. 2). Along with reduced pERK levels the stimulation of miR-221 by loading was dimished suggesting that load-induced miR-221 induction depends on pERK1/2 signaling.

In order to search for prominent pathways comprising putative miR targets, KEGG analysis was performed using all 8 mechanosensitive miRs upregulated by loading (miR-221-3p; miR-1229-5p; miR-1275; miR-4459; miR-6723-5p; miR-6872-3p; miR-6891-5p; miR-7150). Prominent putative miR target molecules were

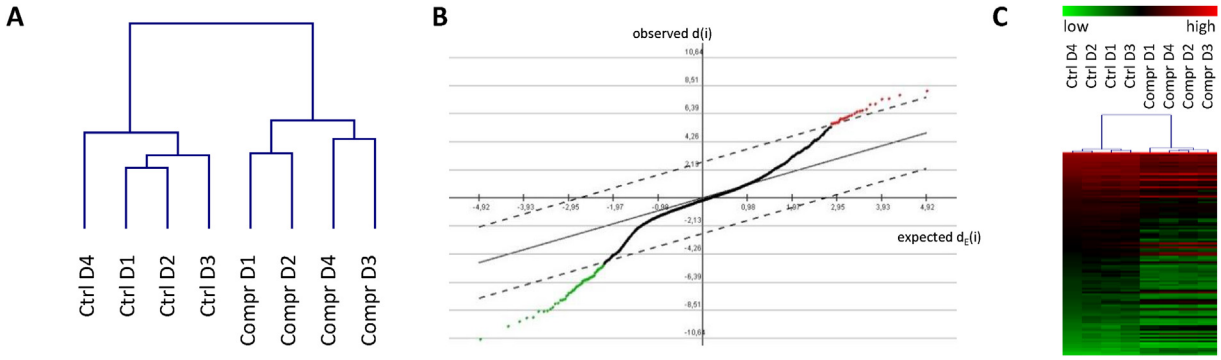

Fig. 3. Global miR expression in response to loading protocol B. Total RNA from eight samples ($n = 4$ per group; four donors) was subjected to miR microarray analysis. A: Hierarchical clustering of normalized and log-transformed expression data without pre-selection. B: Microarray results depicted as scatter plot following data processing by SAM. The observed relative difference d(i) was plotted against the expected relative difference dE(i) for control and compressed groups. Dashed lines define the difference between d(i) and dE(i) beyond which miRs are considered significant at a median FDR < 0.05. In red: probes significantly higher for compressed vs control group, green probes significantly lower in compressed vs control group. C: Hierarchical clustering and heat map visualization of significantly regulated probes. Normalized and log-transformed expression data were color-coded by their level of expression. Green: low expression. Red: high expression.

Table II
Top 20 significantly regulated miRs between controls and compressed samples according to miR microarray analysis from protocol B

microRNA	Ctrl ± sd		Compr ± sd		Fold
hsa-miR-7150	**176.87**	**+/− 37.56**	**682.26**	**+/− 187.22**	**3.86**
hsa-miR-1275	**142.82**	**+/− 13.70**	**526.21**	**+/− 129.35**	**3.68**
hsa-miR-1229-5p	**128.66**	**+/− 19.01**	**340.53**	**+/− 78.75**	**2.65**
hsa-miR-6891-5p	**141.57**	**+/− 18.98**	**370.66**	**+/− 78.35**	**2.62**
hsa-miR-4459	**656.20**	**+/− 73.67**	**1699.38**	**+/− 365.94**	**2.59**
hsa-miR-6125	352.36	+/− 70.03	816.31	+/− 49.78	2.32
hsa-miR-663a	78.60	+/− 6.57	182.02	+/− 35.13	2.32
hsa-miR-3679-5p	97.25	+/− 5.69	196.35	+/−24.79	2.02
hsa-miR-135a-3p	83.91	+/− 3.29	168.87	+/−31.83	2.01
hsa-miR-2392	67.54	+/− 4.90	135.66	+/− 26.15	2.01
hsa-miR-361-5p	199.03	+/− 10.67	126.69	+/− 7.82	−1.56
hsa-miR-19a-3p	165.17	+/− 14.37	103.16	+/− 12.69	−1.61
hsa-miR-19b-3p	189.70	+/− 22.87	117.43	+/− 9.30	−1.61
hsa-miR-199a-5p	2513.93	+/− 238.15	1537.02	+/− 216.27	−1.64
hsa-miR-99a-5p	**1223.87**	**+/− 129.41**	**737.63**	**+/− 118.23**	**−1.67**
hsa-miR-424-5p	**243.37**	**+/− 15.75**	**145.77**	**+/− 15.19**	**−1.67**
hsa-miR-30e-5p	**225.56**	**+/− 16.48**	**128.51**	**+/− 16.80**	**−1.75**
hsa-miR-101-3p	**246.15**	**+/− 26.76**	**133.12**	**+/− 15.29**	**−1.85**
hsa-miR-15a-5p	**596.54**	**+/− 56.71**	**322.29**	**+/− 36.60**	**−1.85**
hsa-miR-199b-5p	**856.20**	**+/− 84.52**	**399.95**	**+/− 55.34**	**−2.13**

$n = 4$ per group; bold, miRs re-analysed by qRT-PCR.

connected to ECM-receptor interaction, cell adhesion molecules, Hippo signaling relevant for TGF-β-, BMP-, WNT-pathway and glycosaminoglycan biosynthesis (Suppl. Table 3). Overall this suggests a functional connection to the identified mechano-miRs to basic mechanisms of load transduction and invites further studies on miR-target identification.

MiR expression in healthy vs OA cartilage

To test whether, in indication of mechanical overuse of OA tissue, at least part of the mechanosensitive miR-pattern may be reproduced in late OA cartilage samples compared with healthy cartilage tissue, mechanosensitive miR-patterns were determined in 20 human healthy cartilage and 21 OA samples by qRT-PCR. None of the PCR-confirmed mechano-miRs upregulated by protocol B (miR-1275, miR-1229-5p, miR-6891-5p) or both loading protocols (miR-6872-3p, miR-6723-5p, miR-221) were significantly higher expressed in OA samples nor was miR-199b-5p significantly suppressed in OA cartilage as seen after overstimulation with protocol B (Fig. 6). Several miRs have previously been reported to be differentially expressed in OA vs normal cartilage and two were also studied with our samples. While elevated expression of miR-675 in OA cartilage was detected in line with Steck *et al.*, 2012,[41] elevation of miR-140-5p[19,21] could not be confirmed. When the three miR-675 outliers in the healthy cartilage group were removed, miR-1275 was significantly elevated in OA samples. Overall, this demonstrated no enduring deregulation of mechanosensitive miRs in articular cartilage of OA patients vs healthy controls.

Discussion

Limited information on mechanosensitive miRs impedes our understanding of basic mechanisms of mechanotransduction in articular cartilage relevant for the pathophysiology of OA. This study provides a global miR expression screen after mechanical challenge of human tissue-engineered cartilage by beneficial and non-beneficial loading conditions provoking either an anabolic or catabolic chondrocyte response. Although anabolic dynamic compression stimulated GAG-synthesis, preserved the GAG-content of the tissue and upregulated expression of 115 genes, among them TGF-β-, BMP-, ERK1/2-, calcium- and WNT-signaling response genes,[31] little consistent changes in miR expression levels were obtained. Only two miRs not reported in the mouse genome (miR-6872-3p and miR-6723-5p) were extracted that responded significantly by transient upregulation at small amplitude. Both were also regulated after catabolic loading. Thus, mechanosensitive miRs were not a significant part of the immediate early physiological loading response by the chosen conditions. However, since one single loading episode as applied here is most likely insufficient to induce a permanent change in GAG synthesis, periodic loading over a longer time frame may be needed to induce mechanosensitive miRs shaping the proteome specifically during anabolic loading responses.

In contrast to physiological loading, non-beneficial loading, which is characterized by downregulation of GAG-synthesis, net loss of GAG from the tissue and reduction of SOX9, ACAN and COL2A1 gene expression, had a broad effect on miR expression with 80 significantly altered miRs; 75% of which were downregulated. This allowed us to define a group of mechanosensitive miRs characteristic for non-beneficial dynamic loading and to extract six miRs reacting only to this catabolic protocol, indicating that these miRs represent markers for loading in a non-physiological range. We can only speculate that in order to prevent permanent damage under non-physiological mechanical

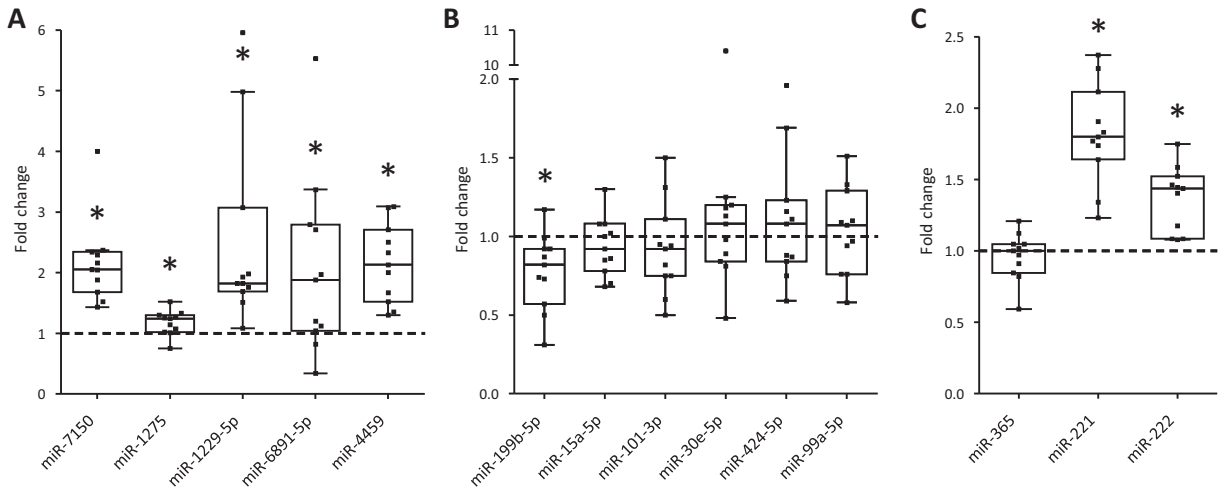

Fig. 4. Expression levels of selected miRs according to qRT-PCR in samples subjected to loading protocol B. Total RNA was isolated from control and compressed samples and miR expression was determined by qRT-PCR. A: Expression levels of miRs that were upregulated according to microarray analysis. B: Expression levels of miRs that were downregulated according to microarray analysis. C: Expression of miRs suggested to be mechanosensitive in chondrocytes in previous studies. Values were normalized to snRNU6 (%U6) and non-loaded samples were set as 1 (dashed line). Data are shown as box plots as indicated in Fig. 1. Mann–Whitney U test, * $P < 0.05$, $n = 11, 11$ donors.

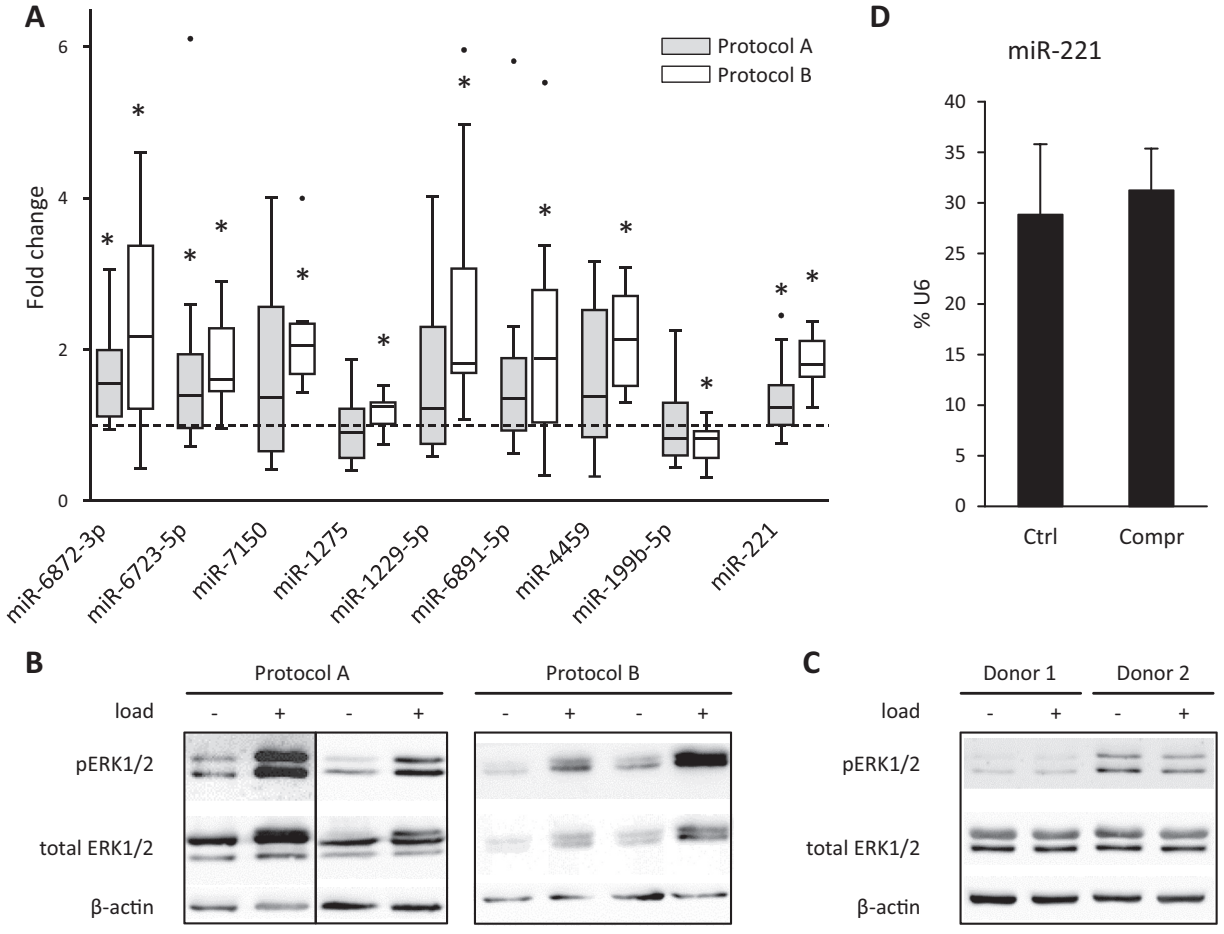

Fig. 5. Cross-testing of mechanosensitive miRs and activation of pERK1/2-signaling. A: Regulation of miR expression was determined after loading with protocol A and loading with protocol B. Expression values were normalized to snRNU6 and non-loaded samples were set as 1 (dashed line). Data are shown as box plots as indicated in Fig. 1. Outliers are depicted as black square. Mann–Whitney U test, * $P < 0.05$, $n = 10–17, 7–14$ donors. B: Western blot analysis of pERK1/2 levels in response to loading with protocol A ($n = 4$, three donors) or loading with protocol B ($n = 3$, three donors). Representative results of two donors are depicted. C: Western blot analysis of pERK1/2 levels in samples on day three of pre-culture with or without exposure to loading with protocol A ($n = 4$, four donors). Total ERK and β-actin levels were used as reference. Representative results of two donors are depicted. D: Expression of miR-221 in day three samples with or without exposure to loading protocol A. Values were referred to snRNU6 (%U6) (mean ± SD; $n = 3$, two experiments).

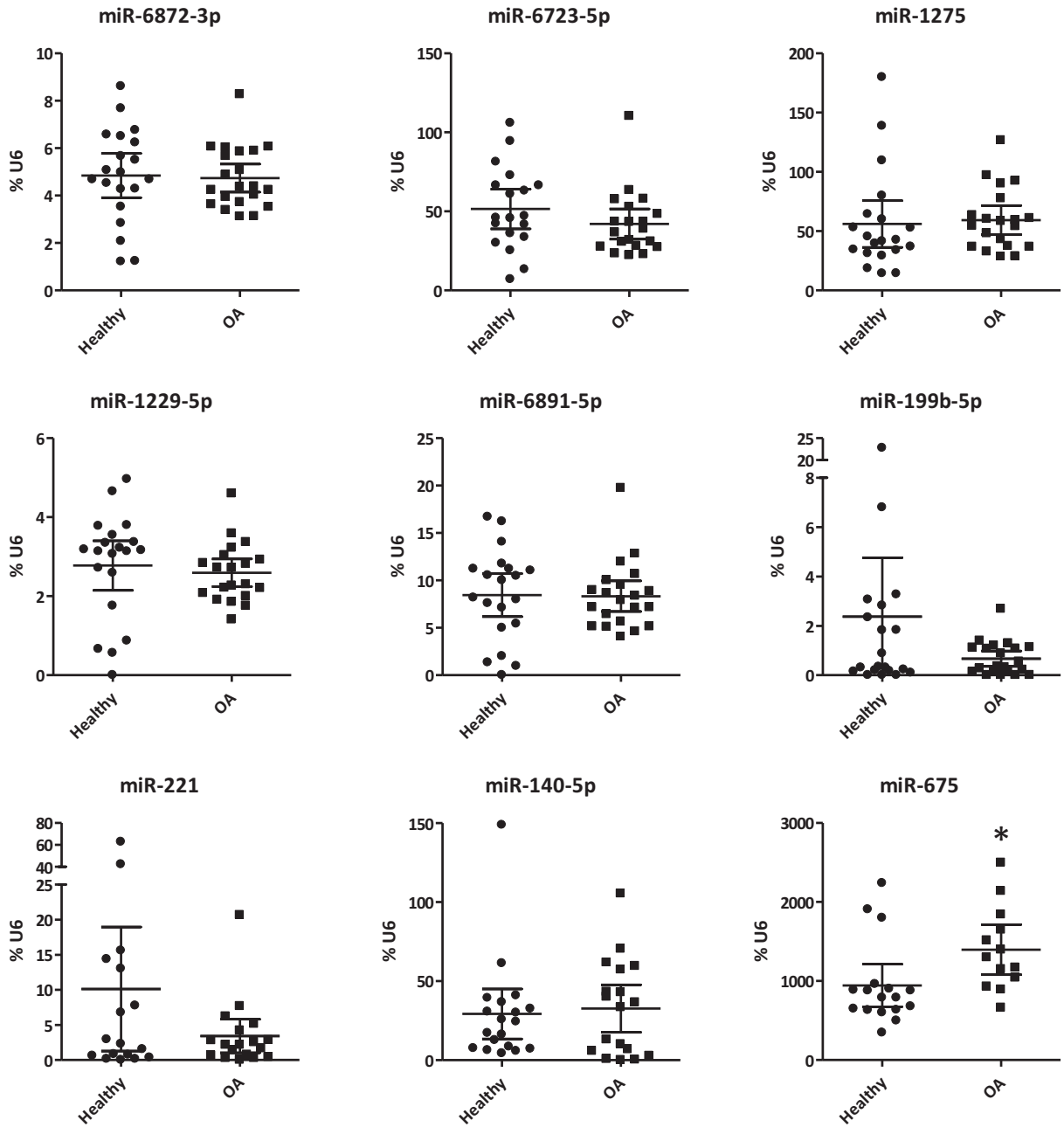

Fig. 6. MiR expression levels in healthy and OA cartilage. Total RNA was isolated from healthy and OA cartilage samples and miR expression was determined by quantitative polymerase chain reaction. Values were referred to snRNU6 (%U6). MiR-7150 and miR-4459 could not be evaluated due to unavailability of primers. $n = 20–21$ OA cartilage, $n = 20$ healthy cartilage. Insufficient total RNA was available from some samples and testing for miRs not extracted by array analysis was only possible in reduced number of tissue samples (miR-221: $n = 18$ OA cartilage, $n = 17$ healthy cartilage; miR-140-5p: $n = 18$ OA cartilage, $n = 19$ healthy cartilage; miR-675: $n = 13$ OA cartilage, $n = 17$ healthy cartilage). The mean value is indicated by a line ±95% confidence intervals are given. Mann–Whitney U test, * $P < 0.05$.

challenge, the cells may activate critical stress response pathways which are shaped by miRs while no such tight epigenetic miR control may be required for anabolic pathways. MiRs for which a narrower mechano-sensitivity was recorded in here may just be induced in association with such a stress response while miRs with a broader mechanosensitivity like miR-221 may be triggered whenever basic mechanotransduction mechanisms are activated irrespective of later outcome. Indeed, miR-221 was only induced when the prominent mechanotransduction pathway pERK was activated by loading and miR-221 stimulation was attenuated when this pathway was inhibited.

Based on the rationale that OA develops frequently in response to overloading, we evaluated the diagnostic value of the discovered mechano-miRs for discrimination of healthy from OA cartilage but no significant differences were observed. It is possible that, such differences have not been recognized due to the limited number of cartilage samples tested in here or that a corresponding enduring change in regulation of mechano-miRs in human OA vs healthy articular cartilage was not found since miR-responses are transient. However, this may change in future studies in which samples can be selected by joint region to separate high load-bearing areas (often completely degraded in human OA samples) from low load-

bearing areas of the joint according to a previous study.[29] After this first demonstration of broad miR regulation in response to catabolic loading of cartilage tissue, *in vivo* studies on common OA animal models are appealing as a next step.

The previously suggested mechanosensitivity of miR-365 observed in chondrocyte-seeded collagen sponges as well as load-induced regulation of its target HDAC4[42] remained unconfirmed in our study. The differences may be explained by mechanical challenge without pre-cultivation and the application of different loading conditions in the previous work.

Among the three miRs regulated by both loading protocols (miR-6872-3p, miR-6723-5p, miR-221) only the function of miR-221 has been described. MiR-221 is species-conserved and expressed in many cells including chondrocytes, skeletal muscle, vascular smooth muscle and cancer cells.[39,40,43,44] MiR-221 was proposed to be regulated by mechanical stress and maximum contact pressure in bovine articular cartilage due to differential expression in load-bearing vs non-load-bearing tissue.[29] Our data now for the first time demonstrate that miR-221 is indeed mechanosensitive but was regulated only when the most prominent mechanotransduction pathway MAPK/pERK1/2 was stimulated, suggesting miR-221 is under the control of pERK1/2 signaling. Although most targets of miR-221 may remain unknown, a frequently proposed target is the cell-cycle-inhibitor p27[39,40,43,44] whose mRNA expression was, however, not regulated in our studies. P27-controlled cell proliferation may be no issue in response to cartilage loading but protein levels need to be judged before a final conclusion can be drawn. Interestingly, the 3′UTR of mRNA of human α10-integrin, an important part of the cell surface mechanoreceptor α10β1, which is signaling via pERK1/2, contains a seed sequence for miR-221 recognition stimulating studies into a potential regulation of α10-integrin in response to loading.

Among the miRs regulated specifically by non-beneficial loading, miR-7150 and miR-6891-5p are largely undescribed. MiR-1275 was involved in IGF-signaling by directly targeting IGF-binding proteins in hepatocellular and squamous cell carcinoma[45,46] while miR-1229-5p contributed to the pathogenesis of occupational noise-induced hearing loss by repressing MAPK1 signaling.[47] MiR-4459 altered stemness maintenance in human embryonic stem cells[48] and miR-199b-5p, highly conserved among species, was so far mostly studied in cancer cells. Among other targets, the Notch-response gene HES1[49] and HIF1α[50] were proposed as target genes for miR-199b-5p. HES1 mRNA and HIF1α protein levels were not significantly changed by loading in our samples (data not shown) and relevant target genes for miR-199b-5p remain to be identified. In line with our results, miR-199b-5p was downregulated 1.6-fold in a miR-array screen of MC3T3 cells exposed to static compression in monolayer[51] but regulation was not further characterized. Overall, expression in chondrocytes and their sensitivity to mechanical challenge is novel for these deeper characterized protocol B-specific miRs. It will now be interesting to correlate miR responses with mRNA expression, identify relevant targets and functional tests will be necessary to decide about the relevance of the seen regulations of single miRs and respective signaling pathways in further studies.

In conclusion we identified the first group of mechanosensitive miRs regulated by cyclic unconfined compression in human chondrocytes and demonstrated that catabolic loading involved a broad shift of miR-levels allowing definition of a “non-beneficial” core-set of miRs (miR-7150, miR-1275, miR-1229-5p, miR-6891-5p, miR-4459, miR-199b-5p) which were only regulated when the tissue responded by inhibition of matrix synthesis. While miRs specific for anabolic loading could not be defined, miRs with broader mechanosensitivity were, including miR-221, which is of great interest because its response was associated with activation of the classical mechanotransduction pathway pERK1/2 and its predicted recognition site in α10β1-integrin as an important mechanoreceptor on chondrocytes. Bioinformatic prediction of putative targets suggested a functional connection of the identified mechano-miRs to basic mechanisms of load transduction and invites further studies on miR-target identification.

Author contributions

NH: Collection and assembly of data, analysis and interpretation of the data, drafting the article, final approval. BJ: Providing of study material, analysis and interpretation of the data, revising the article, final approval. PA, TW: Providing of study material, study conception, revising the article, final approval. WR: Study conception and design, analysis and interpretation of the data, drafting the article, final approval.

Conflict of interest

The authors have no competing interests to declare.

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Supplementary data

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