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### IDENTIFICATION AND CHARACTERISATION OF MICRORNAS INVOLVED IN CHONDROCYTE DIFFERENTIATION AND OSTEOARTHRITIS

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**Purpose:** The majority of skeletal bones develop through the process of endochondral ossification. Mesenchymal cells aggregate where future bones will develop. These early chondrocyte cells begin a series of differentiation events, including proliferation, hypertrophy, terminal differentiation, mineralization and programmed cell death. Many of the signalling pathways and transcription factors which control this developmental programme have been established. MicroRNAs (miRNAs) have emerged as a new class of gene expression regulators. MiRNAs are 20-24 nucleotide non-coding RNA molecules that post-transcriptionally regulate gene expression. Little is known about miRNA expression or function in chondrocyte differentiation. In this study we aimed to profile expression of miRNAs in a cell model of chondrocyte differentiation, verify expression of key miRNAs *in vivo* and investigate function.
**Methods:** The ATDC5 murine embryonic carcinoma cell line was induced to differentiate through chondrogenesis *in vitro*. An Exiqon miRNA microarray was used to profile the expression of all known murine miRNAs across this cell model. Expression of regulated miRNAs was verified in the mouse and chick embryo by *in situ* hybridisation (ISH) using locked nucleic acid probes. To investigate the role of key miRNAs we performed database searches to identify potential targets. 3' UTRs of potential targets were subcloned downstream of a luciferase gene for experimental validation in either SW1353 or C3H10T1/2 cell lines. Expression of key miRNAs was also measured in normal and osteoarthritic cartilage using quantitative RT-PCR.
**Results:** ATDC5 cell differentiation was verified via GAG staining and measurement of key markers by qRT-PCR (e.g. type II and X collagens). In the expression screen of 609 murine miRNAs, we identified 23 miRNAs which were significantly regulated. Of these, we have pursued miR140 and miR455 for further analyses. The expression of both these microRNAs increased across the time course of differentiation just ahead of hypertrophic markers. We and others have previously shown miR140 to be cartilage-specific. Mir455 is located in an intron of a cartilage collagen gene, Col27a1. ISH shows miR455 is expressed in the developing chick and mouse embryo in the developing skeleton. We (Dalmay) have recently shown that miR140 targets Smad3 to decrease TGFβ signalling. Like miR140, miR455 diminishes Smad-dependent signalling to a $(CAGA)_{12}$-luciferase construct. Furthermore, the expression of both miR140 and miR455 is induced by TGFβ1, TGFβ3 and activinA. Preliminary experiments suggest that mir455 targets Smad2 and the type II activin receptor, acvr2b. Both miR140 and miR455 are also increased in expression in human osteoarthritic cartilage compared to normal.
**Conclusions:** A number of microRNAs are strongly regulated during chondrocyte differentiation. At least miR140 and miR455 have the potential to modulate Smad signalling in the growth plate and to function as regulators of chondrocyte proliferation and hypertrophy during endochondral ossification. The increase in expression of miR140 and miR455 in human osteoarthritic cartilage may (i) diminish Smad signalling and/or (ii) reflect or regulate the recapitulation of the chondrocyte developmental programme, contributing to pathogenesis.

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### CARTILAGE-SPECIFIC MICRORNA-140 REGULATES TISSUE HOMEOSTASIS AND PROTECTS AGAINST OSTEOARTHRITIS-LIKE PATHOLOGY

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**Purpose:** MicroRNAs (miRNAs) are a class of non-coding RNAs that negatively regulates gene expression by promoting mRNA degradation and/or repressing translation through partial sequence-specific interactions with the 3' untranslated regions (UTRs) of specific mRNA targets. MiRNAs show tissue specific expression patterns, suggesting that these miRNAs play a crucial role in tissue specific physiological processes and also in human diseases. We previously observed that miR-140 has an expression pattern suggestive of a role in chondrocyte differentiation and found that reduced miR-140 expression in human OA cartilage. Reduced miR-140 expression in human OA cartilage prompted us to determine whether miR-140 functions in cartilage homeostasis. The objective of this study was to define the in vivo function of the chondrocyte specific miR-140 in cartilage homoestasis.
**Methods:** To examine the functions of miR-140, we created a mouse deleted for miR-140 and miR-140 TG mouse, and collected knee joints and chondrocytes from these mice. To examine OA-like pathological changes in articular cartilage, we utilized three different animal models of osteoarthritis: an aging model, a surgical model, and an antigen-induced arthritis (AIA) model. We used a validated histological scoring system based on Safranin O staining and evaluated expression of cartilage related genes by immunohistochemistry. In vitro proteoglycan catabolism was analyzed in cultured femoral head cartilage explants from wild-type, miR-140 TG and miR-140-/- mice. To identify target genes of miR-140, DNA microarray analysis and bioinformatic search were performed. Double-strand (ds) RNA oligos representing mature sequences that mimic endogenous miR-140 were transfected into chondrocytes. Finally, target gene 3' UTR which includes a putative miR-140 binding site was cloned downstream of a luciferase expression vector and the luciferase activity was measured.
**Results:** OA-like pathology in miR-140-/- mice: Postnatally, miR-140-/- mice manifested a mild skeletal phenotype, with short stature and craniofacial deformities characterized by a short snout and domed skull. First, we tested whether loss of miR-140 affected age-related onset of OA changes, and observed that miR-140-/- mice developed an age-related OA-like pathology. Consistent with observations in the aging OA model, the surgical model also demonstrated that miR-140-/- mice exhibit accelerated OA-like changes in knee joints compared with the wild type. Next, to examine whether miR-140 level in articular chondrocytes affects cartilage sensitivity to experimental challenge, we assessed AIA model. Although miR-140-/- mice showed reduced Safranin O staining, importantly, miR-140 TG mice were resistant to proteoglycan and type II collagen loss compared with wild-type mice.
Target gene of miR-140: Identification of miR-140 target genes could provide new insight into miR-140 function and OA pathogenesis. DNA microarray analysis was performed on RNA samples, and many genes were up-regulated in miR-140-/- chondrocytes compared to wild-type chondrocytes. Adamts-5 emerged as one of strong candidate for miR-140 regulation, and its expression was significantly increased in miR-140-/- mice chondrocytes, and this correlated with increased Adamts-5 protein expression in articular cartilage as seen by immunohistochemistry. Cartilage explants from miR-140-/- mice showed significantly increased proteoglycan release compared to wild-type cartilage. Treatment of chondrocytes with ds-miR140 reduced Adamts-5 expression. Luciferase data indicate that miR-140 directly regulates Adamts-5 expression.
**Conclusions:** OA-like changes in miR-140-deficient mice can be attributed, in part, to elevated Adamts-5 expression, regulated directly by miR-140. We show that miR-140 regulates cartilage development and homeostasis, and its loss contributes to the development of age-related OA-like changes.

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### THE MICRORNAS AS BIOMARKERS SPECIFIC OF KNEE OSTEOARTHRITIS

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**Purpose:** We are lacking biomarkers predictive of diagnosis and disease progression in OA. MicroRNAs are small RNAs of 21-23 nucleotides able to inhibit gene expression. The objective of this work is to identify the original miRNAs as biomarkers in two different chronic bone and joint diseases, osteoarthritis (OA) and RA.
**Methods:** Serum samples and fresh blood were obtained from 21 patients with severe knee osteoarthritis (score of Kellgren/Lawrence at least 3/4), 8 RA patients and 10 healthy donors as controls. We performed RNA extraction and qRT-PCR by TaqMan microRNA kit. We analyzed the systemic expression of miRNAs in the 3 groups of patients using microRNA microarrays (Miltenyi). We identified the predictive targets of miRNAs using the DIANA-microT software.
**Results:** OA patients had a mean age of 71 years, had a mean radiological score of Kellgren/Lawrence of 3.8 and their pain has evolved over 7 years. We identified 37 miRNAs in RA and 18 miRNAs in severe knee osteoarthritis