INTRODUCTION:
MiRNAs are small (~22 nucleotide) non-coding RNAs that have been implicated in the regulation of normal cell function. To date we know very little about the expression of miRNAs in articular cartilage. Modulation of miRNA expression is seen in several diseases, including cancer and cardiac disease, and frequently associated with the response to cell stress. By analogy we should expect miRNAs to play a role in the regulation of chondrocyte function associated with cartilage damage. To examine miRNA expression by articular chondrocytes we have adapted RNA extraction protocols that are efficient but unlike widely used protocols for chondrocyte miRNA extraction, recover the small RNAs that include miRNAs. To examine the response to cell stress we have compared miRNA expression in freshly dissected articular cartilage with miRNA expression after chondrocyte isolation and brief culture.

METHODS:
Articular cartilage dissected from adjacent regions of veal hock joints was used for RNA isolation or preparation of chondrocytes. Chondrocytes were isolated by collagenase digestion in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum for 16h, allowed to recover in DMEM 10% fetal calf serum for 24h and cultured under either the same serum or serum-free (DMEM) conditions for 24h. Chondrocytes were also dedifferentiated by 3 sequential subcultures on plastic in DMEM 10% fetal calf serum. For isolation of total RNA chondrocytes were extracted directly whereas cartilage was snap frozen and powdered in liquid nitrogen prior to traditional acid guanidine thiocyanate-pellet extraction followed by proteinase K digestion. RNA quality was assessed by A260/280 and A260/230 ratios. The presence of distinct 28S, 18S and 5S bands was used as a measure of RNA quality and presence of small RNA including miRNA. The small RNA fraction containing miRNA was lost if the high salt guanidine thiocyanate technique commonly used for isolating cartilage mRNA was employed. Two different techniques were used to identify miRNA species. The first employed miRNA purified from total RNA by agarose gel electrophoresis with a micro fluidic array platform (LC Sciences, Houston, TX); the second a locked nucleic acid (LNA) microarray using 2 different array platforms even though the technique and sensitivity currently uncharacterized miRNAs showed greater than 3 fold differences in response to chondrocyte isolation (Figure 1). Changes in expression of this group of miRNAs were confirmed by RTPCR. Semi quantitative endpoint RTPCR (Figure 2) was consistent with results of quantitative real-time PCR. Mir-140 and mir-765 equal expression decreased with dedifferentiation it has been shown to be expressed by many cell types. Mir-140 and mir-483-5p show some specificity to the chondrocyte phenotype, whereas mir-765 had equal expression in dedifferentiated chondrocytes. From predicted target genes and studies in other tissue it appears that the upregulated miRNAs play a role in the regulation of pathways associated with cell division. The known miRNAs shown to be down-regulated have predicted gene targets associated with matrix, MMP and growth factor pathways. These changes would be consistent with a response to the stress of dissociation of the chondrocyte from its matrix. Prediction of miRNA targets from sequence analysis, however, is very imprecise. The function of cartilage miRNAs will require ongoing analysis of the response to enhanced expression using transfection of the miRNA or repression using antimir inhibition.

RESULTS:
The techniques employed allowed efficient isolation of high quality miRNA. Approximately 100 of the more than 1000 miRNA analyzed (described in the Sanger microRNA database Mirbase, http://microrna.sanger.ac.uk ) were shown to be robustly expressed by chondrocytes and could be detected in the tissue or isolated chondrocytes. Minimal differences in miRNAs were apparent using the different array platforms even though the technique and sensitivity differed significantly. MiRNA expression was very consistent between cartilage and chondrocyte preparations. Only five (2 upregulated and 3 down regulated) strongly expressed miRNAs were detected (all down regulated) currently uncharacterized miRNAs showed greater than 3 fold differences in response to chondrocyte isolation (Figure 1). Changes in expression of this group of miRNAs were confirmed by RTPCR. Semi quantitative endpoint RTPCR (Figure 2) was consistent with results of quantitative real-time PCR. Mir-140 has been previously been shown to be strongly expressed in cartilage (3). Like most miRNA strongly expressed in cartilage, no change was seen with chondrocyte isolation. Upregulation of mir-21, mir-221, and down regulation of mir-765, mir-483-5p and, mir-665 occurred concomitantly with down regulation of several matrix and metalloproteinase genes seen after chondrocyte isolation and brief culture. Expression of miRNA-140, miRNA-21 and miRNA-483-5p was substantially decreased with dedifferentiation of chondrocytes (Figure 2).

DISCUSSION:
Articular chondrocytes express a large array of miRNAs. It is likely some of these play a critical role in the regulation of the functions and development of articular cartilage. We have shown that isolation and brief culture of chondrocyte results in changes in a small set of miRNAs. This was accompanied by changes in matrix and MMP gene expression. Loss of expression of miRNAs with dedifferentiation provided some indication of chondrocyte-specific expression. Although mir-21 expression decreased with dedifferentiation it has been shown to be expressed by many cell types. Mir-140 and mir-483-5p show some specificity to the chondrocyte phenotype, whereas mir-765 had equal expression in dedifferentiated chondrocytes. From predicted target genes and studies in other tissue it appears that the upregulated miRNAs play a role in the regulation of pathways associated with cell division. The known miRNAs shown to be down-regulated have predicted gene targets associated with matrix, MMP and growth factor pathways. These changes would be consistent with a response to the stress of dissociation of the chondrocyte from its matrix. Prediction of miRNA targets from sequence analysis, however, is very imprecise. The function of cartilage miRNAs will require ongoing analysis of the response to enhanced expression using transfection of the miRNA or repression using antimir inhibition.