Differentially Expressed MicroRNAs in Developing Human Cartilage: Potential Functions in Regulating Chondrocyte Differentiation

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Introduction: MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that regulate gene expression at the post-transcriptional level. They play critical roles in regulating cellular functions and their dysregulation has been linked to a number of diseases including cancers and cardiovascular disorders. Notably, two reports have shown a link between miRNAs and human skeletal disease (1, 2), thus highlighting an important role for miRNAs in controlling cartilage development and homeostasis. Utilizing laser capture microdissection and microarray technology, we recently reported on highly expressed and differentially-expressed miRNAs in chondrocytes of the developing human limb (3). From this work, specific miRNAs have been selected to determine potential functional roles in regulating chondrocyte gene expression and chondrocyte differentiation. Here we report on four miRNAs that we have identified as being differentially expressed between precursor, differentiated and hypertrophic chondrocytes in vivo. Preliminary studies using TGF-β induced human bone marrow-derived mesenchymal stem/stromal cells (MSCs) suggests functional roles for these miRNAs in regulating chondrocyte differentiation and/or cartilage gene expression patterns.

Methods: To identify differentially expressed miRNAs in chondrocytes of developing human cartilage, laser capture microdissection was carried out on tissue sections of embryonic day 54-56 limbs. At this time point of development, three areas of cartilage could be distinguished containing precursor chondrocytes (PC), differentiated chondrocytes (DC) and hypertrophic chondrocytes (HYP). Following RNA extraction, miRNA expression was determined using TaqMan® OpenArrays® (Life Technologies). A full list of all differentially-expressed miRNAs and more details on the methods used to obtain this expression data are included in our recent publication (3). A number of these differentially expressed miRNAs were then pursued further. Expression of miRs-138, 146b, 196b and 1290 was analyzed in human bone marrow-derived MSCs during TGF-β3-induced chondrogenic differentiation in 3D pellet cultures. Here, RNA was extracted at different time points (Day 0, 2, 4, 7, 14, 21 and 28) using a standard Trizol protocol and expression of each miRNA was analyzed using specific TaqMan® human miRNA assays (Life Technologies). Expression of each miRNA was normalized to RNU44. To over-express each of the four mature miRNAs in vitro, miRIDIAN miRNA mimics (20µM, ThermoFisher) were transfected into MSCs using Lipofectamine RNAiMax transfection reagent in Opti-MEM medium for three days prior to formation of the 3D pellet cultures. Transfection was visually confirmed in monolayer MSCs transfected with the miRNA mimic Dy-547 control. Transfected or non-transfected MSC pellets cultured in the presence of TGF-β3 differentiation medium were then harvested at either day 3 (to confirm miRNA over-expression by TaqMan® miRNA assays) or at day 10. Day 10 pellets were imaged using a Leica dissecting microscope and then processed for RNA extraction to determine expression levels of various chondrocyte genes. Fold changes in gene expression were determined relative to non-transfected control samples.

Results: The selected miRNAs to pursue further were found to be differentially expressed between precursor chondrocytes (PC), differentiated chondrocytes (DC) and hypertrophic chondrocytes (HYP) in cartilage of human developing limbs (3). miR-138: DC > PC (~12 fold); HYP > PC (~16 fold). miR-146b: PC > DC (~4 fold); PC > HYP (~7 fold). miR-196b: PC > DC (~2 fold); DC > HYP (~2 fold); PC > HYP (~5 fold). miR-1290: HYP > DC (~3 fold); HYP > PC (~3 fold). Figure 1 shows expression patterns of these four miRNAs in human bone marrow derived MSCs during TGF-β3-induced differentiation. Expression of miR-138 decreased over time during differentiation. Expression of miRs-146b, 196b and 1290 was more variable during MSC differentiation with highest expression levels (relative to day 0) found at day 7. The highest level of increased expression during TGF-β3-induced differentiation was found for miR-146b (~13 fold at day 7). Preliminary studies using mimics to over-express each of the four mature miRNAs in MSC pellet cultures showed some differences compared to non-transfected controls. Figure 2 shows that the overall pellet size was reduced at day 10 over-expression of either miR-138 or miR-196b. Expression of COL2A1, SOX 9 and COL10A1 was reduced by over 2 fold in the miR-138-transfected cultures at day 10. However, miR-196b over-expression resulted in increased COL2A1 and ACAN expression but a decrease in COL10A1 expression. miR-146b over-expression resulted in increased pellet size, a decrease in COL2A1 expression but a 2 fold increase in COL10A1 expression at day 10. miR-1290 over-expression did not show apparent differences in overall pellet size compared to non-transfected control pellets at day 10. However, in addition to a decrease in COL2A1 and ACAN expression, an increase (over 2 fold) in RUNX2 expression was found in miR-1290-transfected cultures.

Discussion: Although miR-138 expression was found to be higher in differentiated and hypertrophic chondrocytes when compared to progenitor chondrocytes in vivo (3), preliminary data shown here shows decreased expression levels as MSCs become more differentiated and that over-expression of this miRNA in MSCs may inhibit chondrogenesis. The reason for the discrepancy between in vivo and in vitro findings will be pursued further. Gene expression data from miR-196b over-expression
suggests that this miRNA may enhance chondrocyte differentiation but inhibit hypertrophy. Alternatively, results from miR-146b and miR-1290 suggest that these miRNAs may enhance the hypertrophic chondrocyte pathway. The effect of longer-term (i.e. 28 days) over-expression of these miRNAs will be done by generation of lentiviral vectors for transduction into MSCs. Chondrocyte genes found to be decreased by miRNA over-expression may either be direct targets of the miRNA or the miRNA may target other genes (i.e. transcription factors) that normally promote expression of the cartilage gene. Alternatively, those genes found to increase in response to miRNA over-expression (i.e. COL2A1, COL10A1, RUNX2) may be due to the miRNA targeting a transcriptional repressor of that gene. The mechanism of action of these miRNAs (i.e. what genes / cellular pathways are affected) will be pursued further once a functional role in regulating chondrogenesis has been concluded.

Significance: Acquiring knowledge on the functional roles of non-coding miRNAs in regulating chondrocyte differentiation and cartilage homeostasis will be critical for the generation of novel miR-based strategies to treat trauma-induced cartilage defects and prevent progression toward osteoarthritis (OA), the most common cartilage degenerative disease in humans. In addition, identifying miRNAs that can negatively or positively regulate hypertrophic chondrocyte differentiation will be important for treatment of OA as well as endochondral bone fracture repair.

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Figure 1. Expression of miRs-138, 146b, 196b and 1290 during TGF-β3 induced differentiation of human MSCs in pellet culture. For each time point, fold change in expression relative to expression levels at Day 0 is shown.

Figure 2. Effect of miRNA mimics on TGF-β3-induced differentiation of MSCs. Upper panel shows representative images of MSC pellets following over-expression of miRs-138, 146b, 196b or 1290 at day 10 of culture. Scale bars = 0.5mm. CTL = non-transfected control. Lower panel shows changes in expression of some cartilage genes at day 10 following over-expression of each of the four miRNAs. Fold change in gene expression is relative to non-transfected CTL.

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