

GWAS-implicated MicroRNA 199a-5p Regulates Osteoblastic and Chondrogenic Fate of Mesenchymal Stem/Progenitor Cells

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INTRODUCTION: Genome wide association study (GWAS)-implicated bone mineral density (BMD) single nucleotide polymorphisms (SNP) have been shown to localize in cis-regulatory regions of distant effector genes using 3-dimensional functional genomics. Detailed characterization of implicated genes in human primary mesenchymal stem/progenitor cell (MSC) has shown biased terminal differentiation fate of cells, in part due to metabolic and immunologic reprogramming. Among several loci where effector genes are unresolved, the heel ultrasound BMD GWAS-implicated *DNM3* locus is particularly intriguing because the four proxy SNPs rs1992549, rs1992550, rs2586393 and rs6694378 of the sentinel SNP rs12041600 interact with the promoters of non-protein coding genes, a long non-coding RNA *DNM3OS* and the embedded microRNA *MIR199A2* (miR-199a-5p). Although mice with *Dnm3os* disruption shows significant downregulation of *Mir199a* and several skeletal abnormalities after birth including craniofacial hypoplasia, defects in dorsal neural arches and spinous processes of the vertebrae, and osteopenia, the precise role of these genes in osteochondral fate specification of human cells remains elusive.

METHODS: We characterized the temporal expression pattern of implicated genes (*DNM3OS* and miR-199a-5p) at the *DNM3* locus during bone morphogenetic protein-2 (BMP2)-mediated osteoblastogenic and transforming growth factor β -1 (TGF- β 1)-mediated chondrogenic differentiation of MSC. Gene expression patterns were compared with the expression profiles of key transcription factors and marker genes. The functional relevance of the implicated genes for osteochondral fate specification was evaluated by over-expressing *DNM3OS* small interfering RNA (siRNA), a microRNA mimic (miR-199a-5p-mimic) or a microRNA inhibitor (miR-199a-5p-inhibitor) in three independent human MSC donor lines. Gene modified cells were assayed for osteoblastogenic or chondrogenic differentiation at various intervals and changes in the morphological appearance of transfected cells, propagation of differentiating signals, metabolism of key osteoblastogenic and chondrogenic transcription factors, and expression of marker genes were correlated with gene modification.

RESULTS: During BMP2-mediated human MSC osteoblast differentiation, both *DNM3OS* and miR-199a-5p expression (Figure 1A and 1B) temporally decrease and are correlated with the induction of osteoblastogenic transcription factors RUNX2 and Osterix. Although the effect of *DNM3OS* siRNA was minimal on terminal osteoblast differentiation, cells over-expressing miR-199a-5p-mimic depicted a cobblestone-appearance morphological change and ultimately failed to produce extracellular matrix mineralization in the presence of BMP2 (Figure 1C-1E). Mechanistically miR-199a-5p mimic modified cells still propagated BMP/SMAD signaling and expressed osteoblastogenic transcription factors RUNX2 and Osterix, but depicted pronounced upregulation of SOX9 (Figure 1F-1G) and enhanced expression of essential chondrogenic genes *ACAN*, *COMP*, *COL10A1*. Mineralization defect (Figure 1H and 1I), morphological changes and enhanced chondrogenic gene expression associated with miR-199a-5p mimic over-expression could be restored with miR-199a-5p inhibitor suggesting specificity of miR-199a-5p in hMSC chondrogenic fate specification.

The expression of both the *DNM3OS* and miR-199a-5p (Figure 2A) temporally increased and correlated with chondrogenic differentiation (Figure 2B) of hMSC in both monolayers and 3-dimensional pellet culture formats. Although miR-199a-5p mimic over-expression failed to further enhance chondrogenesis in pellet cultures (Figure 2B), blocking miR-199a-5p activity significantly reduced chondrogenic pellet size, extracellular matrix deposition and blunted chondrogenic gene expression (Figure 2C-2E). The effect of miR-199a-5p on chondrogenesis was not due to impaired cell viability because overall DNA content was comparable across transfected groups.

DISCUSSION: Collectively, these data suggest that miR-199a-5p embedded within the long non-coding RNA *DNM3OS* regulates hMSC osteoblast/chondrocyte terminal fate by contextual changes in its expression levels. This is important work as BMP can induce either chondrogenesis or osteogenesis, and discovery of factors that define terminal fate is crucially important for cartilage and fracture repair. Sustained miR-199a-5p activity abrogates hMSC osteoblastogenesis whereas chondrogenesis can not continue in its absence, evidenced by lack of chondrogenic capacity in the presence of miR-199a-5p-inhibitor. miR-199a-5p appears to be a critical regulator of SOX9 expression and possibly functions by downregulation of a *SOX9* transcriptional repressor, which we are testing. Future work will seek to understand the mechanisms of miR-199a expression and to define the mRNA targets of miR-199a.

SIGNIFICANCE/CLINICAL RELEVANCE: Positively or negatively targeting *MIR199A2* could be used to direct MSC fate to enhance cartilage or bone repair.

