Suppression of in vitro osteogenesis by miR-127-3p

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INTRODUCTION: Proper regulation of osteogenesis is essential for bone tissue development and homeostasis. Among the many factors that are required for efficient osteoblast differentiation and mineralization, non-coding microRNAs (miRNAs) are now known to play an important role (1). We previously reported miRNA expression profiles in chondrocytes during long bone development. Among the miRNAs found to be more highly expressed in hypertrophic chondrocytes compared to less differentiated chondrocytes was miR-127-3p (2). This miRNA has also been associated with low bone mass in osteoporosis (3). However, there remains a knowledge gap on how miR-127-3p regulates osteoblast differentiation and the mechanisms involved. In this study, we modulated the activity of miR-127-3p in human bone marrow derived stromal cells (BMSCs) to determine the effects on osteogenesis, and carried out RNA-Seq and biological pathway analyses to gain mechanistic insights.

METHODS: Three hBMSC lines derived from male donors aged 22-25 years were commercially obtained (Lonza Biosciences). Cells were cultured in growth media [low glucose DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 ng/ml basic fibroblast growth factor (bFGF) and antibiotics], expanded to passage 3-4 and utilized for subsequent experiments. hBMSCs were seeded in 24-well plates (100,000 cells/well) and transfected with mirVana® miR-127-3p mimic, miR-127-3p antagomir, or negative control RNA (30 nM), using Lipofectamine™ RNAiMAX. After 24 hours, the culture medium was changed to osteogenic medium, and osteogenesis was conducted for 10 days. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining at day 7, mineral nodule-like formation at day 10 by Alizarin Red staining, and osteogenic marker gene expression at days 7 and 10 by RT-qPCR (SYBR green-based assays for *RUNX2*, *SP7* (osterix), *COL1A1*, *SPP1* (osteopontin), *BGLAP* (osteocalcin) and *IBSP* (bone sialoprotein). Gene expression was normalized to *PPIA*. Expression of miR-127-3p was evaluated by RT-qPCR (Taqman MicroRNA Assays for hsa-miR-127-3p and hsa-miR-127-5p). MiRNA expression was normalized to *SNORD44*. For transcriptomic analysis, hBMSCs were treated with miR-127-3p mimics or control RNAs for 7 days in the presence of osteogenic medium. Following total RNA isolation, bulk RNA sequencing (Illumina NovaSeq 6000) was performed followed by differential gene expression and pathway enrichment analyses. Cell proliferation and viability was analyzed by BrdU ELISA and neutral red assays at days 0, 3, 7 and 10 of osteogenesis in 96-well plates. Filamentous actin (F-actin) organization was evaluated by phalloidin staining at days 3 and 7, on glass chamber slides. Experiments with the three different cell lines were conducted independently ($n = 3$) and showed the same response profile. Statistical analyses were performed using one-way ANOVA and Tukey or Dunnett post-hoc tests (GraphPad Prism software).

RESULTS: Firstly, we analyzed the endogenous expression of miR-127 mature strands (3p and 5p) during hBMSCs osteogenesis. Expression of miR-127-3p (functional strand) was considerably higher than miR-127-5p (assumed to be the non-functional strand). However, no significant difference in expression level of miR-127-5p/3p was observed during osteogenic differentiation. Both miR-127-3p overexpression and inhibition following mimic or antagomir transfection were confirmed throughout the osteogenic period. Inhibition of miR-127-3p did not alter ALP staining, mineralization, or osteogenic gene expression compared to the negative control (NC) group. In contrast, miR-127-3p overexpression (Fig. 1A) led to reduced ALP staining (Fig. 1B), mineralization (Fig. 1C), and downregulation of SP7 and IBSP at days 7 and 10, suggesting an inhibitory effect of miR-127-3p on hBMSC osteogenesis. While no obvious changes in F-actin organization were observed following miR-127-3p overexpression, a reduction in cell proliferation was found (Fig. 2). Preliminary analysis of RNA-Seq and pathway enrichment analysis indicates significant downregulation of biological pathways related to cell cycle and cell proliferation, which agrees with the BrdU proliferation assay results.

DISCUSSION: While we achieved robust inhibition of miR-127-3p by antagomir treatment, we did not observe any significant effects on osteogenesis, which may suggest redundancy from other endogenous miRNAs. However, we demonstrated suppression of osteogenesis by mimic-mediated over-expression of miR-127-3p. Data from RNA-Seq and pathway enrichment analyses suggests that this inhibitory function may be due, in part, to anti-proliferative effects of this miRNA. Of note, tumor suppressor / anti-proliferative function has been reported for miR-127-3p in osteosarcoma cells (4). Current studies are focused on deciphering what proliferative pathways / mediators are affected by miR-127-3p. Closer inspection of differential gene expression data obtained from RNA-Seq analysis reveals 95 up-regulated genes and only 5 down-regulated genes (2-fold change cut-off) due to miR-127-3p over-expression at day 7 of osteogenesis. Among the highest significantly up-regulated genes are those encoding *COL9A3* and *HIF3A*, while *ASPN* (asporin) was significantly down-regulated. Interestingly, type IX collagen has been well-described in cartilage tissue, while HIF3A has been associated with a stable chondrocyte phenotype (5). Asporin has been reported to function as an inhibitor of TGF-beta signaling and a suppressor of chondrogenesis (6). It will be interesting to analyze the extracellular matrix composition in these miR-127-3p-induced cell cultures.

SIGNIFICANCE/CLINICAL RELEVANCE: MiR-127-3p has been implicated in conditions like osteoporosis, growth retardation and inflammation-driven bone loss. A better understanding of its role on osteogenic differentiation could lead to new therapies for bone-related disorders, such as heterotopic ossification.

REFERENCES: 1. Hensley AP, McAlinden A. Bone, 2021; 2. McAlinden A et al. PLoS One, 2013; 3. An JH et al. J Bone Miner Res, 2014; 4. Fellenberg J et al. Cancers (Basel), 2019; 5. Markway BD et al. J Orthop Res, 2015; 6. Kizawa H et al. Nat Genet, 2005.

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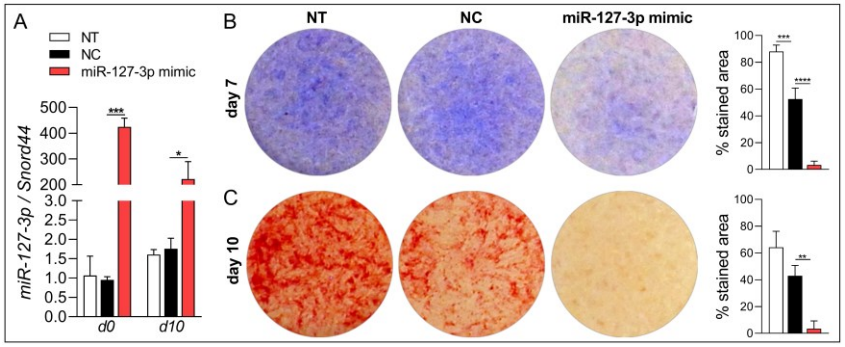

Fig. 1. RT-qPCR for miR-127-3p **(A)**; Alkaline phosphatase **(B)** and alizarin red staining **(C)** of hBMSC following in vitro osteogenesis. *p<0.05, ***p<0.001, ****p<0.0001

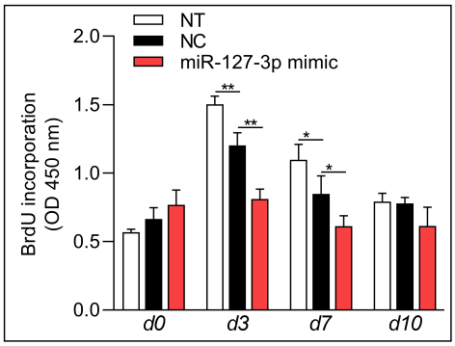

Fig. 2. BrdU incorporation during hBMSC osteogenic differentiation. *p<0.05, **p<0.01