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# miR-342-3p elevates osteogenic differentiation of umbilical cord mesenchymal stem cells via inhibiting Sufu *in vitro*

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ABSTRACT

Human umbilical mesenchymal stem cells (UCMSCs) have been wildly used in tissue engineering field as a promising source because of their unlimited and noninvasive origin. To selectively induce osteogenic differentiation of UCMSCs, it's imperative to understand the regulatory molecular mechanism underlying the process of how these cells switch into osteogenic lineage path. We previously showed enhanced sonic hedgehog (Shh) signaling pathway upon osteogenic induction in mesenchymal stem cells. In this study, miRNA-seq analysis revealed substantial Shh-dependent expression of distinct miRNAs, including miR-342-3p, during ostogenesis. RT-qPCR confirmed that miR-342-3p was increased at a greater level when Shh signaling pathway was activated by N-terminal of Shh ligand compared with osteogenic induction alone, in contrast to the decreasing of suppressor-of-fused protein (Sufu). Consistently, transient overexpressing miR342-3p in UCMSCs via miR-342-3p mimics dramatically decreased Sufu, a suppressor of Gli, while osteogenic markers, including alkaline phosphate and osteocalcin, were upregulated during osteogenic induction, indicating that miR-342-3p might be involved in osteogenesis through the Shh signaling pathway. In conclusion, this study showed the potential of miR-342-3p as a therapeutic target to promote bone regeneration by modulating expression of Sufu in UCMSCs.



## 1. Introduction

Millions of patients suffer from bone loss diseases caused by injury, inflammation, neoplasm, and congenital disorders and excess bone resorption caused by osteoporosis, bone metastasis and periodontitis [1–3]. By restoring contour and functions of impaired bones, quality of life can be significantly improved. In this study, we are trying to understand the molecular mechanisms underlying how stem cells are differentiated into bone forming cells.

Mesenchymal stem cells (MSCs) with osteogenic potential can be isolated from perinatal tissues such as umbilical cord, amniotic fluid, and umbilical cord blood [4,5]. The human umbilical cord MSCs (UCMSCs) have aroused great interest in the last decade because they are derived from a postnatal organ that is discarded after birth [6,7]. Most importantly, these cells share common surface markers with bone marrow-derived mesenchymal stem cells (BMSCs), which are most extensively characterized as they are accepted as “gold standard” of MSCs, researches have shown that UCMSCs possess multipotent properties of prenatal and postnatal MSCs [8,9] and that UCMSCs demonstrate a better capacity of mineralization once exposed to osteogenic induction *in vitro* [10]. Furthermore, global transcriptomic profiling suggested that genes that are related to proliferation were upregulated, whereas HLA-antigens were downregulated, in UCMSCs compared with BMSCs [11,12]. Given those characteristics, UCMSCs are considered to be a promising source for stem cell-based tissue engineering and clinical treatments for bone defects [12–14].

Sonic hedgehog (Shh) signaling pathway plays a critical role in bone development, homeostasis and, diseases [15]. Suppressor of fused protein (Sufu), identified as a suppressor of Shh pathway, is a negative regulator of transcription factor Gli [16,17]. Previous studies have shown that enhanced Shh signaling promotes osteogenesis in various bone-remodeling cells both *in vitro* and *in vivo* [18–21]. A recent study showed that mitogen-activated protein kinases (MAPKs) signaling was involved in UCMSCs during osteogenic differentiation *in vitro* [22]. The role of Shh signaling pathway, however, in bone formation remains unclear.

miRNAs are a family of small non-coding RNAs and are post-

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transcriptional regulators of gene expression in many biological processes including self-renewal, development, and metabolism of stem cells. A number of studies indicated distinct expression profiles of miRNAs in stem cells undergoing osteogenesis [23–25], suggesting the regulatory role of miRNAs during osteogenic differentiation. Although, miRNAs have been shown to regulate cellular development and pathogenesis via targeting Shh signaling mediators [26,27], the association between the profiles of miRNA and Shh signaling pathway during osteogenesis in MSCs has not been fully elucidated. In this study, we investigated the role of Sufu in response to activation of Shh signaling in UCMSCs during osteogenic differentiation *in vitro*. Furthermore, our results revealed a distinct role of miR-342-3p in promoting osteogenesis by down-regulating the expression of Sufu in UCMSCs.

## 2. Materials and methods

### 2.1. Isolation and culture of UCMSCs

This study was ethically approved by Tongji Hospital of HUST university, Wuhan, China. UCMSCs were isolated from umbilical cords obtained after Caesarean section and stored in the cold PBS solution containing 1% penicillin-streptomycin followed by the protocol established by Wang et al. [28]. In brief, umbilical cord was cut into 2 cm pieces and opened lengthwise. After scraping off covering epithelium layer, the blood vessels were removed from the Wharton's jelly. Thereafter, Wharton's Jelly was submerged in 200U/mL collagenase II/PBS (Sigma) solution and incubated at 37 °C overnight. Supernatant was collected and transferred into T25 culture flask next day by additional 6 mL DMEM/F12 (Invitrogen) with 10% FBS (Hyclone). Non-adherent cells were removed by initial medium changed after 24 h. Change the culture medium every other day and pass the cells when observing a >90% confluence.

### 2.2. Multipotency of isolated UCMSCs

UCMSCs were induced to differentiate into adipocytes and osteoblasts as described before [7]. For adipogenic differentiation, cells were seeded at a density of $8 \times 10^4$ cells/cm$^2$ in stem cell medium. The adipogenic induction medium consisted of DMEM high glucose supplemented with 1 mM dexamethasone, 0.2 mM indomethacin, 0.01mg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 10% FBS (Sigma). The medium was changed every 3–4 days for 21 days. After that, cells were fixed with 90% ethanol and stained for 10min with Oil Red O solution (Sigma) to visualize lipid vacuoles. For osteogenic differentiation, cells were seeded at $3.1 \times 10^4$ cells/cm$^2$ as a single cell suspension onto a gelatin-coating plate, and cultured in osteoblastic differentiation mediums, containing osteogenic supplements as follows: 50 μg/mL ascorbic acid (Sigma), 10 nm dexamethasone (Sigma), and 5 mm β-glycerophosphate (Sigma), respectively. The osteogenic medium was changed every 3 days and the cells were collected at 3, 7, 14 days. After 14 days of differentiation, cells were fixed with 95% ethanol for 1 h, washed with PBS and stained with Alizarin red solution (Sigma) for 10min. Cells were washed with PBS and calcium deposits were visible. To activate Shh signaling pathway, additional 1.7 μg/mL of Shh-N was added with or with osteogenic medium to treat UCMSCs for 7 days.

### 2.3. Flowcytometry

Supplemental methods.

### 2.4. RT-qPCR

Supplemental methods.

### 2.5. miRNA sequencing analysis

Three biological replicates of UCMSCs were pooled from each treatment (vehicle, OI and Shh-N) to perform miRNA-seq analysis. The RNA molecules in a size range of 18–30 nt were enriched by polyacrylamide gel electrophoresis. Then the 3′ and 5′ adapters were added and the 36-44 nt RNAs were enriched. The ligation products were reverse transcripted by PCR amplification and the 140-160bp size PCR products were enriched to generate a cDNA library and sequenced using Illumina HiSeq™ 2500 by Gene Denovo Biotechnology Co. (China). The raw data were processed using Illimina Genomi Analyzer and analyzed as previously described by Liu et al. [29]. The differentially expressed miRNA across the treatments was computed by algorithm suggested by Ernstet et al. [30]. miRNA with fold change $\geq$1.5 and $p < 0.05$ in comparison was considered as the significant differentially expressed miRNA.

### 2.6. miRNA-342-3p transient transfection

To over-express miRNA-342-3p in UCMSCs, cells were transfected with miR-342-3p precursor hairpin, negative control pre-miR (Ambion) using siPORT NeoFX transfection reagent (Ambion) according to the manufacture's instruction. Briefly, miR-342-3p or negative control was diluted into 50 μL Opti-MEM I medium at a concentration of 30 nM and mixed with diluted siPORT NeoFX transfection reagent. After 10min incubation at room temperature to allow transfection complexes to form and subsequently dispensed into wells of a clean 6-well culture plate. The UCMSCs were suspended in DMEM (Invitrogen) with 10% FBS (Hyclone) and overlaid onto the transfection complexes. Transfected cells were incubated at 37 °C and 5% $CO_2$ for 24 h.

### 2.7. Protein analysis

Supplemental methods.

### 2.8. Statistics

Statistical analyses were carried out using GraphPad Prism (GraphPad). All results are reported as Mean ± SD of three independent experiments performed in triplicates. Statistical comparison between two groups was performed using Student's t-test. When comparing more than two groups One-Way Analysis of Variance (ANOVA) test was used followed by Tukey's posttest or two-way ANOVA with Bonferroni's posttest were used, with $p < 0.05$ considered to be statistically significant.

## 3. Results

### 3.1. Activation of Shh signaling during osteogenic differentiation in UCMSCs

UCMSCs were successfully collected and propagated from umbilical cords obtained after Caesarean sections (N = 3). They revealed a consistent phenotype, which was positive for mesenchymal markers (CD 105, CD146, CD44 and CD29) ranging from 89% to 100% and negative (<1%) for hematopoietic markers (CD14, CD34) (Fig. 1A). After cultured in osteogenic medium for 14 days, calcium and hydroxyapatite salts stained in red were found in

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