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MiR-34a Promotes Osteogenic Differentiation of Human Adipose-Derived Stem Cells via the *RBP2/NOTCH1/CYCLIN D1* Coregulatory Network

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SUMMARY

MiR-34a was demonstrated to be upregulated during the osteogenic differentiation of human adipose-derived stem cells (hASCs). Overexpression of miR-34a significantly increased alkaline phosphatase activity, mineralization capacity, and the expression of osteogenesis-associated genes in hASCs in vitro. Enhanced heterotopic bone formation in vivo was also observed upon overexpression of miR-34a in hASCs. Mechanistic investigations revealed that miR-34a inhibited the expression of retinoblastoma binding protein 2 (*RBP2*) and reduced the luciferase activity of reporter gene construct comprising putative miR-34a binding sites in the 3′ UTR of *RBP2*. Moreover, miR-34a downregulated the expression of *NOTCH1* and *CYCLIN D1* and upregulated the expression of *RUNX2* by targeting *RBP2*, *NOTCH1*, and *CYCLIN D1*. Taken together, our results suggested that miR-34a promotes the osteogenic differentiation of hASCs via the *RBP2/NOTCH1/CYCLIN D1* coregulatory network, indicating that miR-34a-targeted therapy could be a valuable approach to promote bone regeneration.

INTRODUCTION

Tissue engineering technology has become one of the most promising therapeutic approaches for bone regeneration in bone defects (Zou et al., 2011; Ye et al., 2011; Xiao et al., 2011). As a source of mesenchymal stem cells (MSCs), human adipose-derived stem cells (hASCs) are receiving more attention in bone tissue engineering (Bosnakovski et al., 2005; Zuk et al., 2002; Wang et al., 2011). However, the paucity of available information about the molecular pathways that govern the osteogenic differentiation of hASCs has hampered further development of hASC-based cell therapies.

MicroRNAs (miRNAs) are a class of endogenously expressed, small non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional level by base pairing with the 3′ UTR of their target mRNAs (Thomas et al., 2010). They play vital roles in various biological processes, including the cell fate of embryonic stem cells, cell proliferation, apoptosis, differentiation, morphogenesis, carcinogenesis, and angiogenesis (Ambros, 2004; Hua et al., 2006; Xu et al., 2004). A single miRNA is often involved in several gene regulatory networks (Bartel, 2004; Krek et al., 2005), and overexpression or inhibition of miRNAs can regulate the endogenous expression of multiple growth factors simultaneously (Yau et al., 2012). Therefore, we hypothesized that the delivery of a desired miRNA may result in optimization of bone regeneration. Recent studies have reported that several miRNAs, such as miR-22, -100, -106a, -146a, and -148b, are involved in the osteogenic differentiation of stem cells (Cho et al., 2010; Huang et al., 2012; Li et al., 2013a; Liao et al., 2014; Qureshi et al., 2013; Zeng et al., 2012). However, further regulatory mechanisms of miRNAs in the osteogenesis of MSCs still await investigation.

Our previous study showed that the inhibition of retinoblastoma binding protein 2 (*RBP2*) significantly improved the in vitro and in vivo osteogenic capacity of hASCs (Ge et al., 2011). Based on these data, we aimed to screen and select miRNAs that positively regulate the osteogenic differentiation of hASCs by targeting *RBP2*. Microarray analyses revealed that after osteogenic induction, 21 miRNAs were upregulated in hASCs (Zhang et al., 2012) and 51 miRNAs were upregulated in bone marrow-derived MSCs (BMSCs) (Gao et al., 2011), suggesting that 72 upregulated miRNAs had potential effects on the osteogenic differentiation of MSCs. Moreover, RNA22 prediction software indicated that 122 miRNAs might bind to the 3′ UTR of *RBP2* mRNA. These two categories of miRNAs were combined and an intersection of five miRNAs was produced: miR-663, -34a, -26a, -17, and -155. The RNA22 prediction software predicted their corresponding folding energy (ΔG) was −14.00 kcal/mol, −16.8 kcal/mol, −12.50 kcal/mol, −13.20 kcal/mol, and −13.30 kcal/mol. According to the results predicted

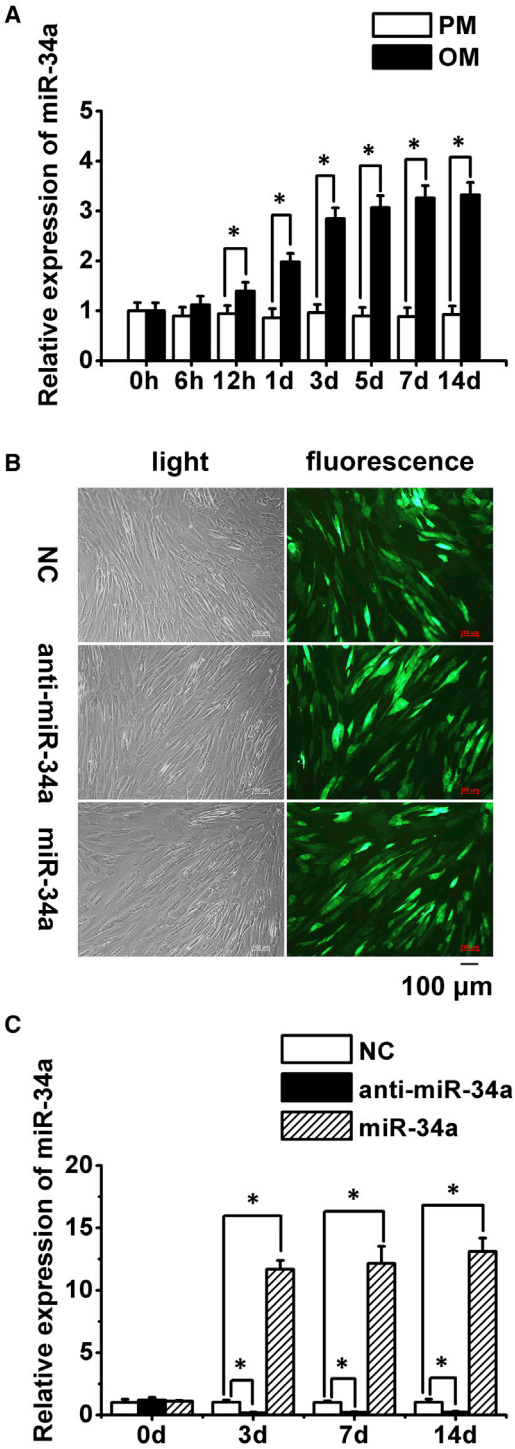

Figure 1. Expression of Endogenous miR-34a during hASCs' Osteogenic Induction, and Determination of Lentiviral Transduction Efficiency and Effect

(A) Quantitative real-time PCR analysis of miR-34a expression in hASCs cultured in PM and OM.

(B) Microscopic images of GFP-positive hASCs under ordinary and fluorescent light. Scale bar, 100 μm.

(C) Quantitative real-time PCR analysis of miR-34a in transduced hASCs cultured in PM.

PM, proliferation medium; OM, osteogenic medium; NC, lentivirus negative control; anti-miR-34a, lentivirus anti-sense miR-34a; miR-34a, lentivirus miR-34a mimics. Data represent the means ± SD of three independent experiments. *p < 0.05 versus the NC group.

by RNA22 prediction software, miR-34a possessed the maximum likelihood for binding to the 3′ UTR of *RBP2* mRNA (ΔG = −16.8 kcal/mol); therefore, we selected miR-34a for further investigation (Figure S1).

NOTCH1 and *CYCLIN D1* are direct target genes of miR-34a in tumor cells (Hermeking, 2010; Pang et al., 2010), and have effects on the proliferation and osteogenic differentiation of MSCs by regulating runt-related transcription factor 2 (*RUNX2*) (Engin et al., 2008), a key osteogenesis-associated transcription factor. Thus, *NOTCH1* and *CYCLIN D1* pathways were integrated into our hypothetical regulatory network of miR-34a.

In this study, we investigated the functional roles of miR-34a in the osteogenic differentiation of hASCs both in vitro and in vivo, and explored whether miR-34a regulated this biological process through the *RBP2/NOTCH1/CYCLIN D1* coregulatory network. Our study provided a better understanding of the role and mechanism of miR-34a in hASCs' osteogenic differentiation and suggested that miR-34a could be a therapeutic target in future bone regeneration therapy, which will lead to advances in clinical bone tissue engineering.

RESULTS

Expression Levels of miR-34a during the Osteogenic Differentiation of hASCs

After culturing hASCs in osteogenic medium (OM) for 12 hr, miR-34a expression increased significantly, and further increased with prolonged osteogenic induction. However, no significant change was detected in hASCs cultured in proliferation medium (PM) when compared with the 0-hr time point (Figure 1A). These data suggested that miR-34a might play a role in the regulation of hASCs' osteogenic differentiation.

Promotion Effects of miR-34a on the Osteogenic Differentiation of hASCs In Vitro

The transduction efficiency of lentivirus was estimated to be 80%–90%, as evaluated by the percentage of GFP-positive cells under an inverted fluorescence microscope 72 hr after transduction (Figure 1B). Quantitative real-time PCR analysis of miR-34a expression in transduced hASCs cultured in PM at 0, 3, 7, and 14 days showed