

Successful use of miRIDIAN™ microRNA mimic and inhibitor libraries identify microRNAs involved in human mesenchymal stem cell osteogenesis

Yuriy Fedorov and Jon Karpilow, Dharmacon, now part of Horizon Discovery, Lafayette, CO, USA

Introduction

MicroRNAs are endogenous non-coding RNAs that modulate gene expression through the RNA interference pathway¹. The mechanism of regulation is generally conserved across nature and is predicted to influence a significant portion of the human genome during early and adult stages of development²⁻⁵. As microRNAs have been shown to play a role in stem cell fate determination, potentially exciting opportunities exist for applications in regenerative medicine.

Human mesenchymal stem cells (hMSC) are capable of differentiating into multiple lineages including bone, cartilage, and tendon⁶. Unfortunately, while these cells can be easily isolated and expanded, little is known about the contributions that microRNAs make to MSC differentiation. Recently, several approaches, including microarray and quantitative real-time PCR (qPCR) technologies, have been used to identify microRNAs that are disproportionately expressed in differentiated and undifferentiated states. Unfortunately, while these approaches can provide potential insights into the role for microRNAs in cell fate determination, they are restricted by two limitations. First, in most in vitro cell differentiation assays only a fraction of the cells undergo differentiation. Thus, in the absence of enrichment by Fluorescence Activated Cell Sorting (FACS) “differentiated” cell profiles are contaminated with those of undifferentiated cells, thereby compromising the ability to identify relevant microRNAs.

A second shortcoming of microarray and qPCR technologies is that these techniques only provide a snapshot of the “before” and “after” cellular states. As it is plausible that microRNAs play a role in the transition from a multipotent to fully differentiated state, experimental strategies that allow investigation of the contribution of microRNAs to all stages of cellular differentiation are necessary. In the following application note, we demonstrate how screening with miRNA mimic and inhibitor libraries can be successfully used to identify microRNAs that play a role in cell fate determination, specifically, hMSC osteogenic differentiation.

Assay workflow

The overall workflow used in these studies is presented in Figure 1. For the initial phase - Assay development – we chose to quantify alkaline phosphatase (AP), an early marker of MSC osteogenic differentiation⁹. Alkaline phosphatase levels were assessed by one of two methods. In the primary screen, cells were lysed and total AP was quantified using the p-nitrophenol phosphate conversion method (BRSC, NY). Alternatively, the fraction of AP positive cells in a population were measured by staining the culture with the fluorogenic ELF97 substrate, and then quantifying images using Thermo Scientific™ ArrayScan™ VTI high content imaging system.

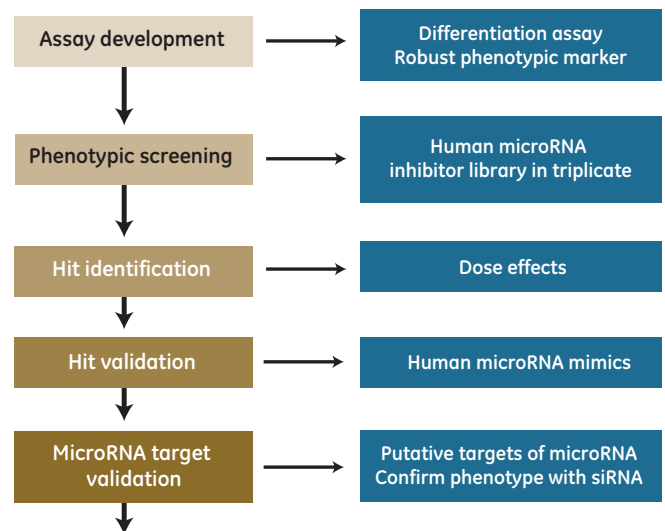


Figure 1. Experimental workflow encompassing assay development, phenotypic screening, hit identification and validation of leads to identification of biologically relevant endogenous targets.

The primary screen utilized Dharmacon™ miRIDIAN™ collection of inhibitors targeting all the known microRNAs of miRBase 8.2. hMSCs (Lonza) were plated in propagation medium and then transfected with the miRIDIAN microRNAs inhibitor collection using Dharmacon™ DharmaFECT™ 1 transfection reagent. The propagation medium was then replaced with a differentiation cocktail and cells were cultured prior to assessing total AP activity (Figure 2). In these studies, negative controls consisted of 1) inhibitors targeting *C. elegans* microRNAs, and 2) a Dharmacon™ SMARTpool™ of Non-targeting siRNAs. As a positive control, a SMARTpool targeting RUNX2, a transcription factor required for hMSC osteogenic differentiation, was employed (see micrograph in Figure 2 for performance of controls).

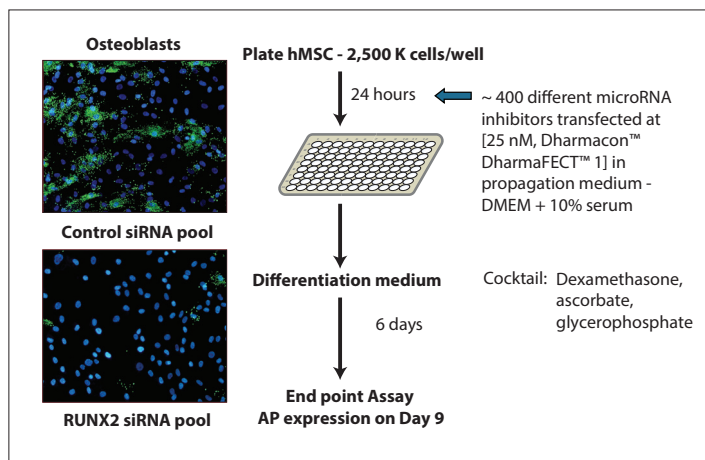


Figure 2. Schematic of human microRNA inhibitor phenotypic screening. Propagation medium = DMEM + 10% FBS. Differentiation medium = propagation medium, dexamethasone, ascorbate, and glycerophosphate. Fluorescent micrographs show the relative performance. Negative (top) and positive (bottom) controls.

Validating hits identified in microRNA inhibitor screens requires approaches that are distinct from those used in siRNA phenotypic screens. For siRNA screens, hit validation involves demonstrating that multiple siRNAs targeting the same transcript induce the same phenotype. As microRNAs are short, 17-28 basepair duplexes, the possibility of designing multiple, non-overlapping inhibitors to each microRNA does not exist. For this reason, validation consisted of 1) eliminating primary screen hits that did not exhibit concentration dependent responses with the inhibitors, and 2) selecting hits in which the matching microRNA mimic and inhibitors pairs induced opposite effects.

Screen and validation results

From the original library of 396 human microRNA inhibitors, 15 molecules (3.8%) significantly altered AP expression in the primary screen (data not shown). Seven of these molecules (inhibitors of miR-189, -153, -133a, -186, -27a, -148b, and -489) induced dose-dependent effects with six increasing AP activity and a single inhibitor (i-148b) significantly decreasing AP activity. Mimics of miR-189, -153, -133a, and -186 microRNAs had no effect on AP activity, suggesting that these genes might be necessary but not sufficient for regulation of early hMSC osteogenesis. In contrast, three microRNA mimics,

miRs-27a, -148b, and -489 significantly affected AP activity in a manner that was opposite of that induced by the matching inhibitor (Figure 3). The opposite phenotypes observed with mimics and inhibitors support the notion that these three microRNAs play an important and essential role in early osteogenic differentiation.

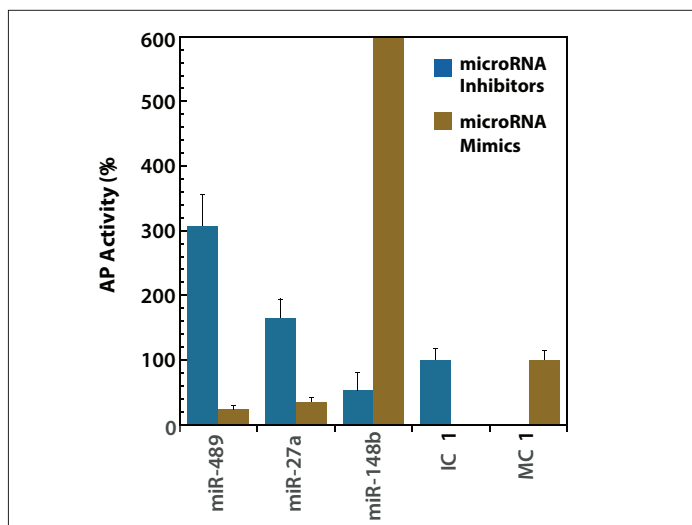


Figure 3. Bar graph showing the opposing effects of mimic-inhibitor pairs in osteogenic differentiation. AP = Alkaline Phosphatase.

Summary

A comparative study using microRNA microarrays identified only two microRNAs that were recognized to be important in the inhibitor screening. Thus, the study presented above clearly demonstrates the validity of using microRNA mimic and inhibitors to identify non-coding RNAs that are essential for biological processes.

References

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If you have any questions, contact

t +44 (0) 1223 976 000 (UK) **or** +1 800 235 9880 (USA); +1 303 604 9499 (USA)

f +44 (0)1223 655 581

w horizondiscovery.com/contact-us **or** dharmacon.horizondiscovery.com/service-and-support

Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

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