APPLICATION NOTE

Distinguishing true microRNA targets involved in stem cell differentiation: integrating technologies.

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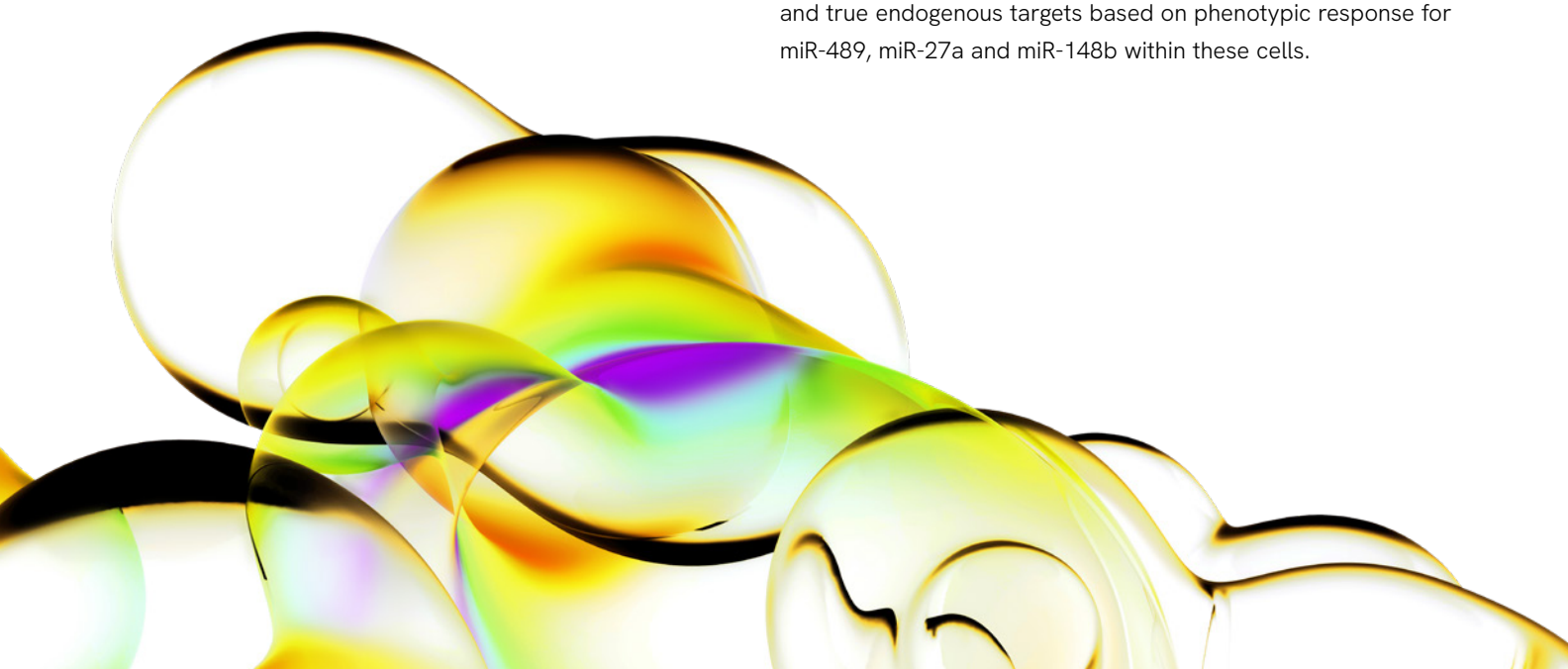
Introduction

MicroRNAs are non-coding RNAs that provide critical translational regulation within the cell[1]. This type of regulation occurs via base pairing of the microRNA (~ 22 nucleotides) to target sites in the 3′ UTR of mammalian protein-coding genes, thus exerting control over a large proportion of the transcriptome[2-5]. Although biochemical methods have been described to help elucidate endogenous targets of microRNAs, often these do not directly address the phenotypic relevance of a microRNA binding event[6]. In addition to their role in normal development and the adult cell population, microRNAs are key regulators of stem cell differentiation making them attractive tools for regenerative therapies[7-9].

Human mesenchymal stem cells are non-hematopoietic, stromal cells that can differentiate into multiple cell lineages. Adult bone marrow-derived hMSCs are easily isolated, expanded and differentiated into a variety of tissues including bone, cartilage, and tendon[10]. Their ease of manipulation, propagation and multipotency make hMSCs a very attractive resource for regenerative therapy and a model system for investigation. A loss-of-function screen was performed using the Dharmacon™ miRIDIAN™ human microRNA inhibitor library revealing key effectors of hMSC differentiation. In addition, application of corresponding miRIDIAN microRNA mimics verified the direct role of these microRNAs in osteogenesis. This approach identified three microRNAs (miR-489, miR-27a and miR-148b) that regulate hMSC differentiation, quantified by the expression of bone-specific alkaline phosphatase. The next step is to distinguish between the predicted targets based on sequence and true endogenous targets based on phenotypic response for miR-489, miR-27a and miR-148b within these cells.



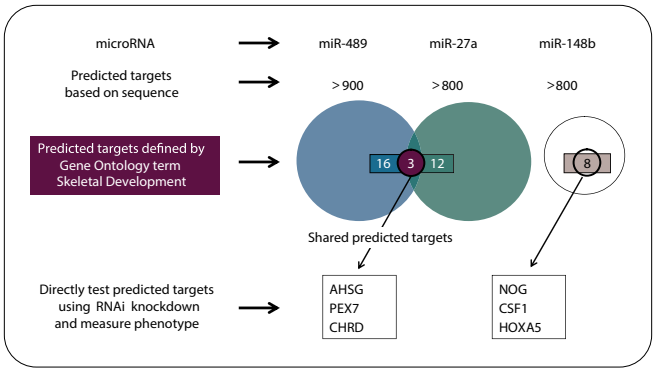


Figure 1: Distinguishing between predicted and true microRNA targets. This Illustration is showing a large number of predicted targets based on microRNA sequence alone using the miRanda algorithm[1]. Using the Gene Ontology term for 'Skeletal Development', a focused list of predicted targets was revealed. Those targets could then be disrupted using target-specific RNAi knockdown to determine involvement in hMSC differentiation into osteoblasts.

Identifying predicted microRNA targets

There are a number of different algorithms available to predict microRNA targets. However, in general there is an over estimation of targets based solely on the sequence content of microRNAs. As might be expected there was a large number of predicted targets based on the sequence content for miR-489 (> 900), miR-27a (> 800) and miR-148b (> 800) as illustrated in Figure 1. Here we used the miRanda algorithm to generate predicted targets based on microRNA sequence.

To narrow down this list we combined a second bioinformatics tool; gene ontology. Specifically, we used a gene ontology analysis term for skeletal development to further identify mRNA transcripts that were likely to be relevant in our model system; hMSC differentiation into osteoblasts. This resulted in the prediction of 16 targets for miR-489, 12 targets for miR-27a and 8 targets for miR-148b which were subsequently prioritized as outlined in Figure 1.

Validating true microRNA targets

To validate the role of each transcript in osteogenic differentiation, thus distinguishing between the predicted and true endogenous targets, Dharmacon™ SMARTpool™ siRNA technology was employed. The involvement of a microRNA in a specific phenotype is likely to be propagated through its interaction with one or more endogenous mRNA targets. Therefore, disruption of the putative mRNA targets with SMART selection designed siRNAs provides an opportunity to directly test the relevance of each target for the specific phenotype being measured. We individually knocked down each of the six predicted endogenous mRNA targets and measured the effect on osteogenesis. When individual siRNA-mediated knockdown was employed, only two of the six predicted targets exhibited phenotypes similar to that already observed with the corresponding synthetic microRNA mimics (Figure 2). Specifically, application of the synthetic microRNA mimic for miR-498 and siRNA-mediated knockdown of Chordin, a known inhibitor of bone morphogenic protein, decreased osteogenic differentiation. In addition, application of the synthetic microRNA mimic for miR-148b and the siRNA-mediated knockdown of Noggin, a known bone morphogenic protein antagonist, increased osteogenic differentiation.

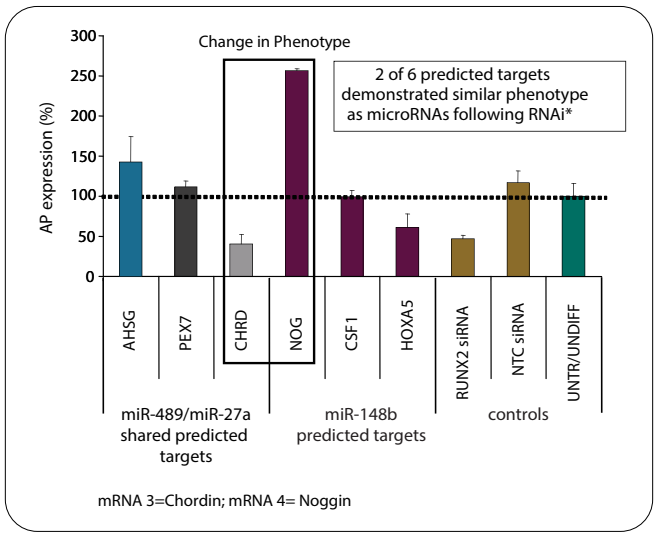


Figure 2: Distinguishing between predicted and true microRNA targets. This graph is showing that when all six predicted target mRNAs are individually knocked down, only two demonstrably alter alkaline phosphatase (AP) expression for example hMSC differentiation. *Predicted target mRNA knockdown was achieved using our SMARTpool siRNAs. AHSG= alpha-2-HS-glycoprotein; PEX7= peroxisomal biogenesis factor 7; CHRD= Chordin; NOG= Noggin; CSF1= colony stimulating factor 1; HOXA5= homeobox 5A; RUNX2= positive control; NTC= non-targeting control SMARTpool (Cat #D-001206-14); UNTR/UNDIFF= untreated/undifferentiated.

Summary

In our initial studies we developed a robust phenotypic assay to test the hypothesis that modulating different microRNAs, using the miRIDIAN microRNA inhibitor library, would identify important regulators of hMSC differentiation. This approach allowed us to identify a number of microRNAs that clearly were involved in the regulation of osteogenesis. By adopting a rational bioinformatics process involving a target prediction algorithm, as well as a gene ontology analysis, we were able to focus on those predicted targets directly associated with skeletal development. Furthermore, we were able to apply SMARTpool siRNA technology to distinguish between the predicted and true endogenous mRNA target transcripts. Thus, a multi-pronged approach encompassing assay development, bioinformatics expertise and microRNA and siRNA technologies has helped to clarify biologically relevant targets that directly influence osteogenic differentiation.

References

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