SPECIAL FOCUS: RNA THERAPEUTICS FOR TISSUE ENGINEERING*

Analysis of -5p and -3p Strands of miR-145 and miR-140 During Mesenchymal Stem Cell Chondrogenic Differentiation

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The chondrogenic differentiation of mesenchymal stem cells (MSCs) is mediated by transcription factors and small noncoding RNAs such as microRNAs (miRNAs). Each miRNA is initially transcribed as a long transcript, which matures to produce -5p and -3p strands. It is widely believed that the mature and functional miRNA from any given pre-miRNA, usually the -5p strand, is functional, while the opposing -3p strand is degraded. However, recent cartilage literature started to show functional -3p strands for a few miRNAs. This study aimed at examining both -5p and -3p strands of two key miRNAs miR-140 and miR-145, known to be involved in the chondrogenic differentiation of MSCs. The level (copy number) of both -5p and -3p strands of miR-145 and miR-140 along the time line of MSC chondrogenic differentiation was determined by polymerase chain reaction. The gene expression profiles of several genes related to MSC chondrogenesis were compared with these miRNA profiles along the same timeline. While miR-145-3p is declining in step with miR-145-5p in pellet cultures during the process, the -3p strand is only 1–2% of the total miR-145 products. In contrast, the mature -3p and -5p products of miR-140 are found to increase with near-equal molar expression throughout chondrogenic differentiation. Numerous genes are expressed by cartilage progenitor cells during development. One such target gene, Sox9, is a regulatory target of the dominant miR-145-5p, consistent with the data. Further experimental validations are warranted to confirm that ACAN, FOXO1, and RUNX3 as direct targets of miR-145-5p in the context of MSC chondrogenesis. Similarly, TRSP1 and ACAN are worth further validation as direct targets of miR-145-3p. For miR-140, SOX4 shall be further validated as a direct target of miR-140-5p, while KLF4, PTHLH, and WNT5A can be validated as direct targets of miR-140-3p.

Keywords: miRNA, 5p versus 3p, chondrogenesis

Introduction

THE REGULATION OF chondrogenic differentiation of mesenchymal stem cells (MSCs) is mediated by both transcription factors and small noncoding RNAs called microRNAs (miRNAs). Understanding the details of this regulation is beneficial for optimizing tissue-engineered (TE) cartilage for repair and regeneration. The translation and stability of messenger RNA (mRNA) transcripts are controlled, in part, by miRNAs through sequence-specific binding to complementary sequences within mRNA transcripts. Once bound, both the miRNA and mRNA are either degraded or blocked from being translated through sequestration.¹

Each miRNA is initially transcribed as a long transcript, which acquires a secondary structure, including one or more stem loops. The stem loops from each pre-miRNA

are subsequently cleaved to produce -5p and -3p mature strands (Fig. 1). While specifics of binding and processing of these fragments are not entirely understood, the mature and functional miRNA from any given pre-miRNA seemed to be designated as one dominant strand and the opposing strand is degraded. Anecdotal evidence generally pointed to the -5p strand as the functional mature miRNA.² Recent studies with cancer cells, however, have revealed a functional significance of the -3p strand.^{3–5} The issue has not been studied intentionally for TE cartilage other than a few sporadic findings related to the -3p strand.^{6,7} Thus, in this Technical Report, both -5p and -3p strands of two key miRNAs known to be involved in the chondrogenic differentiation of MSCs are quantified in an effort to determine if each or both are relevant to chondrogenic differentiation.

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FIG. 1. In this representation of pre-miRNA processing, the protein Dicer binds to a pre-miRNA transcript and then cleaves the stem loop. The Dicer ribonucleoprotein complex then discards either the mature -5p or -3p miRNA fragment and associates with TRBP and Argonaute 2 to form the RISC ribonucleoprotein complex. The RISC ribonucleoprotein complex will finally associate with nearly complementary target sequences in mRNA target genes and degrade or sequester them ultimately blocking translation. miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex.

Numerous genes are expressed by cartilage progenitor cells during cartilage development. Among these, the most often referenced are *TGF- β* , *COL2*, *COL9A1*, *COL11A2*, *ACAN*, *SOX5*, *SOX6*, *SOX9*, *SOX13*, *FOXA3*, *FOXD1*, *FOXO1*, *Foxp2*, *BMP8a*, *WNT4*, *HOXA10*, *HOXD4*, *HOXD9*, *HOXD10*, *HOXD11*, *KLF2*, *KLF4*, *LEPRE1*, *TRPV4*, *SOX11*, *Wnt5a*, and *Wnt5b*.^{8–14} Meanwhile, other genes are involved in hypertrophy (endochondral ossification), osteogenesis, and suppressing chondrogenic differentiation (*COL10*, *COL7*, *CAS3*, *BMP2*, *CTNNB1*, *FGF9*, *FGFR1*, *RUNX2*, *RUNX3*, *BGLAP*, *ALPL*, *MMP-13*, *MMP-9*, *IHH*, *SNAI2*, *SOX4*, *WNT3A*).^{15–21}

Both miR-140 and miR-145 are known to target some of the genes involved in or tightly regulated during chondrogenesis.⁷ One such target gene, *Sox9*, is considered a master regulator of cartilage development and a regulatory target of miR-145-5p.²² *Sox9* is a transcription factor capable of inducing miR-140 expression.²³ Knocking out miR-140 in mice leads to skeletal defects and early-onset osteoarthritic phenotypes suggestive of cartilage homeostasis dysregulation.²⁴

This study was designed to determine the changing expression of the mature strands (5p, 3p) of both miR-145 and miR-140 during the time course of chondrogenic pellet formation. We additionally compare the gene expression profiles (obtained from a previous RNA microarray investigation²⁵) of several genes known to be important during *in vitro* MSC chondrogenesis and potentially targeted by either miR-140 or miR-145. Our study of miRNA expression utilizes copy number analysis allowing a direct comparison of both the -5p and -3p for each miRNA. We demonstrate miR-145-5p expression dominated, while only 1–5% of miR-145 transcripts were miR-145-3p. However, miR-140-5p and miR-140-3p are expressed at nearly equal molar ratios. Future pathway analysis of these miRNA targets would provide additional insight into the contribution(s) of each miRNA strand (-5p and -3p) during MSC chondrogenic differentiation.

Materials and Methods

MSC isolation, culture, and chondrogenic differentiation (pellet cultures)

Human MSCs were obtained from iliac crest bone marrow aspirates provided by four separate consenting donors

and provided by the Case Western Reserve University Skeletal Research Center in compliance with our University Hospitals of Cleveland Institutional Review Board protocol. Donors 1 and 2 were used in the microarray experiments previously reported in Somoza *et al.*,²⁶ while donors 3 and 4 were utilized in the experiments for this report. MSCs were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (complete medium) at 37°C and 5% CO₂ for one passage before generating pellet cultures prepared as previously described.²⁷

The second passage MSCs were used to generate chondrogenic pellets. Briefly, MSC cultures from first passage were trypsinized with 0.25% trypsin EDTA, washed once with complete DMEM, and resuspended in basal pellet medium (DMEM-high glucose [HG] supplemented with 1% ITS+Premix, 100 μ M ascorbate-2-phosphate, 10⁻⁷ M dexamethasone) at 1.25 $\times$ 10⁶ MSC/mL. Next, 0.25 $\times$ 10⁶ (200 μ L) MSCs were added to each well of a 96-well non-adherent poly-propylene V-bottomed plate. The plate was centrifuged for 5 min at 500 g, media removed, and replaced with chondrogenic induction media (basal pellet medium with 10 ng/mL TGF- β 1). Pellet cultures were then incubated at 37°C and 5% CO₂. MSCs for two donors were saved at day 0 and pellet culture samples were harvested on days 3, 7, 10, 14, 21, and 28.

Histological analysis

Pellets collected on days 14, 18, and 26 were fixed in 10% neutral buffered formalin, paraffin embedded, and then ribbon sectioned. Slides were then stained with hematoxylin and eosin or Safranin O by standard protocol by the histological staining service of the Case Western Reserve University Department of Biology Center for Skeletal Research.

Absolute copy number real-time polymerase chain reaction analysis

Chondrogenic pellets obtained from donors 3 and 4 on days 3, 7, 10, 14, 21, and 28 were used to generate total RNA with TRIzol reagent (Cat. No. 15596026; Thermo Fisher) for each pellet according to the manufacturer's protocol. Total RNA

(10 ng for each reaction) was used to generate complementary DNA (cDNA) with the TaqMan[™] MicroRNA Reverse Transcription Kit (Cat. No. 4366596; Applied Biosystems). Absolute copy number real-time polymerase chain reaction (RT-PCR) was performed on 1.33 μ L of cDNA for each reaction with the TaqMan[®] Universal Master Mix II, with UNG (Cat. No. 4440038; Applied Biosystems).

For each RT-PCR reaction, we generated a standard curve with dilutions of a DNA oligonucleotide mimic of each miRNA (miR-145-5p, 5'-GTC CAG TTT TCC CAG GAA TCC CT-3'; miR-145-3p, 5'-GGA TTC CTG GAA ATA CTG TTC T-3'; 5'-CAG TGG TTT TAC CCT ATG GTA G-3'; miR-140-3p, 5'-TAC CAC AGG GTA GAA CCA CGG-3'). The following primers were used to perform in the absolute copy number RT-PCR: TaqMan[®] MicroRNA Assay has-miR-145-5p, Assay ID 002278; TaqMan MicroRNA Assay hsa-miR-145-3p, Assay ID 002149; TaqMan MicroRNA Assay mmu-miR-140-5p, Assay ID 001187; TaqMan MicroRNA Assay hsa-miR-140-3p, Assay ID 002234.

Cycle threshold (Ct) values are the product concentration thresholds used to determine at which cycle a kinetic reaction achieves this concentration. Ideally, a Ct value is chosen within the linear phase of an exponential growth curve so this curve can best estimate the initial copy number of the target RNA. Because each probe has unique reaction kinetics, selection of the optimal Ct value within the linear phase of exponential growth yields different values for each probe. For this analysis, the Ct value selected for miR-145-5p was 0.017799, for miR-145-3p was 0.37554, for miR-140-3p was 0.513477, and for miR-140-5p was 0.040824. Copy number graphs were produced in and linear regression performed with Microsoft Excel software.

Results

Chondrogenic pellet formation

Chondrogenic pellet cultures developed over 28 days. Pellet growth increased and Safranin O staining of proteoglycan is indicative cartilage formation occurred (Fig. 2A, B). Safranin O staining was lighter than expected in these samples. This likely represents batch-specific Safranin O variability rather than poor proteoglycan production.

Expression of mature miR-145 and miR-140 -3p and -5p strands

To determine the expression of mature -3p and -5p strands of miR-145 and miR-140 during MSC chondrogenic differentiation, we quantified miR-145-3p, miR-145-5p, miR-140-3p, and miR-140-5p copy numbers from chondrogenic pellets sampled during MSC chondrogenic differentiation by absolute quantitative RT-PCR (qRT-PCR). Mature strands of miR-145 declined over the 28-day culture period, while miR-140 strands accumulated (Fig. 2C). As expected, miR-145-5p strands were present at every time point at nearly 100 times the abundance of the miR-145-3p strands (Figs. 2C, D, and 3A). By contrast, the ratios of miR-140-5p to miR-140-3p were observed at nearly equal molar amounts (Figs. 2E, F, and 3B). These results suggest regulation of miRNA maturation, and -3p/-5p strand sta-

bility could be important to regulation of chondrogenic differentiation.

Prediction of miR-145 and miR-140 target genes

Since these two miRNAs are known to play important roles in controlling gene expression during MSC chondrogenic differentiation, we utilized the online mircoRNA.org database^{25,28,29} (accessed October 4, 2017) to identify mRNA targets of these miRNAs with known roles in chondrogenesis. The putative chondrogenic and/or transient chondrogenic gene targets of miR-145-3p and -5p and miR-140-3p and -5p are shown in Tables 1 and 2, respectively, with associated PhastCons and mirSVR scores. Consistent with our data, miR-145-5p gene targets include genes previously identified as critical for chondrogenic differentiation (*ACAN*,¹¹ *FOXO1*, *SOX9*, and *SOX11*).^{10,30} In contrast, miR-140-3p and 5p gene targets included genes previously identified as taking part in terminal hypertrophic differentiation resulting in endochondral bone formation (transient chondrogenic differentiation). Previously validated targets of miR-140-3p and 5p include *BMP2*, *CTNNB1*, *FGF9*, *FGFR1*, *RUNX2*, *SNAI2*, and *JAG1*.^{25,28,29,31}

Expression analysis of miRNAs and their target genes

We next compared the mature miRNA -3p and -5p copy numbers for miR-140 and miR-145 on specific days during chondrogenic differentiation to the expression of potential target genes involved in chondrogenic and hypertrophic chondrogenic differentiation (as determined by a previously reported microarray analysis of pellet cultures from two different human donor MSC preparations).²⁶ Published studies have experimentally verified *SOX9*²² as the target of miR-145-5p during MSC chondrogenic differentiation. Similarly, *JAG1*³¹ is an experimentally validated target of miR-140-3p. *JAG1* is expressed in osteoarthritis and is an active suppressor of *SOX9*.^{32,33} Our data show, with the exception of *SOX11*, gene expression of *ACAN*, *FOXO1*, and *SOX9* in the microarray pellets increased, while miR-145-5p copy number declined as would be expected if these genes are suppressed by miR-145-5p (Fig. 4A–E). Expression of genes was involved in transient cartilage development (endochondral bone formation) potentially targeted by miR-145-5p; *SNAI2* and *SOX4* but not *RUNX3* increased with increasing miR-145-5p copy number, as shown in Figure 4A and F–H.

Similarly, potential targets of miR-145-3p, miR-140-5p, and miR-140-3p also potentially target genes involved in chondrogenic differentiation or transient hypertrophic chondrogenic differentiation. *TRSP1* and *ACAN* but not *KLF4*, all with complementary binding site at their 3' untranslated region (UTR) for miR-145-3p, increased as miR-145-3p decreased (Fig. 5A–D). Chondrogenic genes, with complementary binding site at their 3'UTR for miR-140-5p, *BMP8A*, *FOXP2*, *SOX11*, and *WNT4*, did not display strongly inverse expression as would be expected of genes controlled by this miRNA (Fig. 6A–E).

Transient hypertrophic chondrogenic gene potential targets of miR-140-5p, *BMP2*, *SNAI2*, and *SOX4* but not *CTNNB1* or *FGF9*, declined as miR-140-5p copy number increased (Fig. 6A, G–J). The chondrogenic putative gene targets of miR-140-3p, *KLF4*, *PTH1H*, and *WNT5A* but not

FIG. 2. Histological hematoxylin and eosin (A) and Safranin O (B) staining of day 14, 18, and 26 pellets. Copy number quantification of miR-145-5p (C), miR-145-3p (D), miR-140-5p (E), and miR-140-3p (F) expressed in pellets from MSCs obtained from two donors during chondrogenic differentiation. MSCs, mesenchymal stem cells.

FIG. 3. The ratio of miR-145-5p (A) or miR-140-5p (B) copy number to the total copies (total = [miRNA-3p + miRNA-5p]) of each miRNA.

TABLE 1. CHONDROGENIC GENES TARGETED BY MATURE miR-145 AND miR-140 FRAGMENTS

<i>Highest expression</i>		<i>miRNA targets</i>									
<i>Gene</i>	<i>hMSC pellets</i>	<i>NAC</i>	<i>miR-145-5p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>	<i>miR-145-3p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>	<i>miR-140-5p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>
<i>ACAN</i>	291	1428	2×	0.5858	-0.1423	1×	0.6132	-1.0311	—	—	—
<i>BMP8A</i>	109	115	—	—	—	—	—	—	1×	0.5142	-0.1977
<i>FOXO1</i>	1209	3321	1×	0.7412	-1.0000	—	—	—	—	—	—
<i>FOXP2</i>	118	155	—	—	—	—	—	—	2×	0.8019	-1.1064
<i>GLI3</i>	240	348	—	—	—	—	—	—	1×	0.6723	-0.3652
<i>HOXA10</i>	1143	358	—	—	—	—	—	—	—	—	—
<i>HOXD10</i>	133	166	—	—	—	—	—	—	—	—	—
<i>KLF4</i>	1047	415	—	—	—	1×	0.5423	-0.5949	—	—	0.7633
<i>PTH1H</i>	200	276	—	—	—	—	—	—	—	—	0.6831
<i>SOX11</i>	165	135	5×	0.8057	-0.1016	—	—	—	—	—	-0.9258
<i>SOX9</i>	2194	1072	2×	0.5725	-1.0271	—	—	—	2×	0.722	-0.1842
<i>TRPS1</i>	506	158	—	—	—	2×	0.8153	-0.1226	—	—	—
<i>WNT4</i>	195	122	—	—	—	—	—	—	4×	0.5774	-0.0949
<i>WNT5A</i>	2184	111	—	—	—	—	—	—	—	—	—
									4×	0.6229	-0.5935

Highest expression indicates the highest expression observed in the average of pellet culture samples or NAC samples.
hMSC, human mesenchymal stem cell; miRNA, microRNA; NAC, neonatal articular cartilage.

TABLE 2. ANTI-CHONDROGENIC GENES TARGETED BY MATURE miR-145 AND miR140 TRANSCRIPTS

<i>Highest expression</i>		<i>miRNA targets</i>									
<i>Gene</i>	<i>hMSC pellets</i>	<i>NAC</i>	<i>miR-145-5p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>	<i>miR-145-3p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>	<i>miR-140-5p</i>	<i>PhastCons score</i>	<i>mirSVR score</i>
<i>BMP2</i>	1700	373	—	—	—	—	—	—	1×	0.7607	-1.324
<i>CTNNA1</i>	2334	349	—	—	—	—	—	—	1×	0.7438	-0.1002
<i>FGF9</i>	117	137	—	—	—	—	—	—	1×	0.7368	-1.1093
<i>FGFR1</i>	259	350	—	—	—	—	—	—	—	—	—
<i>RUNX2</i>	605	149	—	—	—	—	—	—	—	—	—
<i>RUNX3</i>	837	163	2×	0.5149	-0.1269	—	—	—	—	—	—
<i>SNAI2</i>	3285	1727	1×	0.7415	-1.1721	—	—	—	1×	0.5615	-0.7806
<i>SOX4</i>	2065	255	3×	0.8072	-0.1104	—	—	—	1×	0.7547	-0.6645

Highest expression indicates the highest expression observed in the average of pellet culture samples or NAC samples.

FIG. 4. Copy number quantification of miR-145-5p (A) expressed in pellets from MSC obtained from two donors compared to microarray expression values for miR-145-5p targets ACAN (B), FOXO1 (C), SOX11 (D), SOX9 (E), RUNX3 (F), SNAI2 (G), and SOX4 (H) obtained from two different donors.

HOXA10, decreased with increasing miR-140-5p copy number (Fig. 7A–E). The transient chondrogenic genes likely targeted by miR-140-3p, *BMP2* and *JAG1* but not *FGF9*, *FGFR1*, and *RUNX2*, declined with increasing miR-140-3p copy number (Fig. 7A, G–J).

Discussion

The limited scope of this work demonstrates that the -3p and -5p maturation products of miR-145 differ largely in molar ratio during MSC chondrogenic differentiation, which

FIG. 5. Copy number quantification of miR-145-3p (A) expressed in pellets from MSC obtained from two donors compared to microarray expression values for miR-145-3p targets *ACAN* (B), *KLF4* (C), and *TRPS1* (D) obtained from two different donors.

is not the case for miR-140. While we did observe miR-145-3p declining in step with miR-145-5p in pellet cultures, the -3p strand was only 1% or 2% of the total miR-145 products. This result suggests miR-145-3p plays a minor biological role and/or is somehow actively degraded. Indeed, only 1% of miR-145 products were miR-145-3p in the human melanoma cell line A375 (data not shown), lending credit to the -3p product having little biological significance. In contrast to the miR-145 results, the mature -3p and -5p products of miR-140 demonstrated near-equal molar expression throughout chondrogenic differentiation. However, these results are limited by the small sample number in the study. The findings are nevertheless unexpected and an important step to understanding the consequence that miRNA processing has on modulating gene expression during the differentiation process and thus warranting further study with a greater number of donor samples and inclusion of other potentially relevant miRNAs.

Among putative chondrogenic gene targets of miR-145-5p, *SOX9* was previously experimentally confirmed for MSC chondrogenesis.³⁴ From gene expression profiles shown in Figure 4, perhaps *ACAN* and *RUNX3* are worth further confirming as either the direct or indirect targets of miR-145-5p in the context of MSC chondrogenesis. Clearly, inverse correlation was not observed between *SNAI2* and miR-145-5p expression. *SNAI2* was confirmed as a target of miR-145-5p in which hsa-miR-145-5p transfection in human renal carcinoma (*ACHN*) cells led to reduced expression of *SNAI2* by qRT-PCR.³⁴ However, *SNAI2* may not be a target of miR-145-5p in the context of MSCs during chondrogenic differ-

entiation, where a different microenvironment and regulatory pathways exist. It is, nonetheless, possible that a more complex regulatory control over *SNAI2* is involved.

In contrast to miR-145, both mature strands of miR-140 increase during pellet formation suggesting these mature -3p and -5p products are important to chondrogenic differentiation. This is in line with previous studies that miR-140 indirectly stabilizes *SOX9* and *ACAN* expression as well as suppresses the response to IL1 exposure in differentiating MSCs.^{31,35} In addition, *SOX5* and *SOX6* act in concert with *SOX9* in promoting miR-140 pre-miRNA expression.³⁶ It is conceivable that both miR-140-5p and -3p mature strands accumulate over time, in nearly equal molar ratios, and concurrently with *SOX9* expression.

Similarly, *SOX4* can be further confirmed experimentally as the direct target of miR-140-5p. Likewise, *KLF4*, *PTH1H*, *WNT5A*, and *BMP2* should be further confirmed as either the direct or indirect targets of miR-140-3p. *FGF9* is a confirmed target of miR-140-5p in hepatocellular carcinoma by overexpression and reporter assay.³⁷ However, *FGF9* may not be a target in the context of MSC chondrogenesis. Indeed, an inverse correlation with *FGF9* (target of miR-140-5p) was not observed. In addition, the level of *FGF9* remained significantly low throughout the process indicating that *FGF9* may not be active during MSC chondrogenic differentiation. While these results do not confirm targeting by the -3p and -5p mature products of miR-145 and miR-140, we do show that, in some instances, genes known to regulate the chondrogenic process are expressed as would be expected if regulated by these miRNAs.

FIG. 6. Copy number quantification of miR-140-5p (A) expressed in pellets from MSC obtained from two donors compared to microarray expression values for miR-140-5p targets *BMP8A* (B), *FOXP2* (C), *SOX11* (D), *WNT4* (E), *BMP2* (F), *CTNNB1* (G), *FGF9* (H), *SNAI2* (I), and *SOX4* (J) obtained from two different donors.

FIG. 7. Copy number quantification of miR-140-3p (A) expressed in pellets from MSC obtained from two donors compared to microarray expression values for miR-140-3p targets *HOXA10* (B), *KLF4* (C), *PTHLH* (D), *WNT5A* (E), *BMP2* (F), *FGF9* (G), *FGFR1* (H), and *RUNX2* (I), and *JAG1* (J) obtained from two different donors.

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Disclosure Statement

The authors have no financial relationships that may cause a conflict of interest.

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