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miR-21 Modulates the ERK–MAPK Signaling Pathway by Regulating SPRY2 Expression During Human Mesenchymal Stem Cell Differentiation

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ABSTRACT

The ERK–MAPK signaling pathway plays a pivotal role during mesenchymal stem cell (MSC) differentiation. Studies have demonstrated that ERK–MAPK promotes adipogenesis and osteogenesis through the phosphorylation of differentiation-associated transcription factors and that it is the only active signaling in all three lineages (adipogenic, chondrogenic, and osteogenic) during MSC differentiation. Recent studies pointed to the significant roles of microRNA-21 (miR-21) during several physiological and pathological processes, especially stem cell fate determination. The miR-21 expression pattern is also correlated with ERK–MAPK activity. Here, we found that miR-21 expression is elevated and associated with an increased differentiation potential in MSCs during adipogenesis and osteogenesis. The overexpression of miR-21 elevated the expression level of the differentiation-associated genes PPARγ and Cbfa-1 during MSC differentiation, whereas miR-21 knockdown reduced the expression level of both genes. The ERK–MAPK signaling pathway activity had an increasing tendency to respond to miR-21 upregulation and a decreasing tendency to respond to miR-21 down-regulation during the first 4 days of adipogenesis and osteogenesis. Our data indicate that miR-21 modulated ERK–MAPK signaling activity by repressing SPRY2 expression, a known regulator of the receptor tyrosine kinase (RTK) signaling pathway, to affect the duration and magnitude of ERK–MAPK activity. The ERK–MAPK signaling pathway was regulated by Sprouty2 (SPRY2) expression via a miR-21-mediated mechanism during MSC differentiation. J. Cell. Biochem. 114: 1374–1384, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: MESENCHYMAL STEM CELL; miR-21; ERK–MAPK SIGNALING PATHWAY; SPRY2; ADIPOGENESIS; OSTEOGENESIS; FEEDBACK LOOP

Mesenchymal stem cells (MSCs) are derived from the mesoderm and can differentiate into various mesoderm-type cells during the mesengenic process, such as adipocyte, osteocyte, chondrocyte, and skeletal muscle: these derivatives are also defined as mesenchymal cells [Bianco et al., 2008]. MSCs were commonly recognized for their therapeutic potential in clinical applications and attracted much attention even though the precise regulatory mechanism that underlies MSC fate determination was not yet elucidated. Phosphorylation plays a critical role in cell fate determination [Kratchmarova et al., 2005]. The phosphorylation status of differentiation-associated transcription factors determine the duration and progression of the differentiation process [Edlund and Jessell, 1999]. For example, several studies have reported that the MAPK signaling pathway affected adipogenesis [Bost et al., 2002; Liao et al., 2008] and osteogenesis [Meyers et al., 2004; Ge et al., 2007] during MSC differentiation. Specifically, PPARγ gene expression [Prusty et al., 2002] and Cbfa-1 gene transcription activity [Xiao et al., 2000] are promoted by the phosphorylation of these genes, which are markers of adipogenesis and osteogenesis, respectively [Ducy et al., 1997; Rosen et al., 2000; Franceschi1 and Xiao, 2003]. Furthermore, the precisely controlled mechanism behind the MAPK signaling pathway leads to critical questions regarding its involvement in distinct processes. Positive and negative feedback loops can precisely control signaling pathway in development [Freeman, 2000]. Several MAPK-responsive transcription factors and characteristics of MAPK signaling

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pathway, such as the magnitude and duration of the activity of this pathway, are involved with feedback regulation during cell fate specification.

Recent experiments demonstrated that miRNAs and some genes are components of regulating feedback loops to control signal pathways during development [Johnston et al., 2005; Li and Carthew, 2005]. The contribution of miRNAs to known regulation pathways further elucidates the networks that are involved in cell fate determination [Ivey and Srivastava, 2010]. The miRNA is a noncoding RNA that has a conserved, short (17–24 nucleotides) sequence and has been identified in species ranging from viruses to mammals. miRNAs reduced the level of target protein expression by post-translational regulation by interacting with the cognate mRNA [Bartel, 2004]. The mechanism of the microRNA: target interaction may subtly and modestly influence protein output. As one of the most ancient and conserved interaction models, the microRNA:-target interaction appears to involve a complicated regulation network but not simple linear relationship [Bartel, 2009]. This mechanism of action may relay versatile and complex extracellular signals to the nucleus in a precise and timely manner.

The functions of microRNAs have been increasingly characterized as being associated with development. Several previous studies demonstrated that elevated miR-21 expression is involved in both carcinogenesis [Volinia et al., 2006] and development [Houbaviy et al., 2003]. miR-21 was a differentiation-associated microRNA in embryonic stem cell (ESC) differentiation [Singh et al., 2008], and miR-21 also promoted adipogenesis during MSC differentiation through the TGF-β/Smad signaling pathway [Kim et al., 2009]. However, the miR-21 expression level also increased when MSC differentiated along the osetogenic pathway [Goff et al., 2008]. Our previous data showed that miR-21 expression was upregulated compared with other miRNAs during MSC culture in growth medium (data not shown). A high expression level of miR-21 was the major observable trait in multiple types of cancer cell lines and differentiation stages. The function of miR-21 may account for its self-sustained mechanism in diverse physiological and pathological conditions.

Moreover, understanding the molecular mechanisms that control microRNA expression would further elucidate their function in different processes [Neppl and Wang, 2009]. Some miRNAs expression was regulated by some PDGF-responsive transcription factors during osteogenesis [Goff et al., 2008]. In this report, miR-21 was also identified as an upregulated miRNA during osteogenesis. However, miR-21 transcription was not modulated by any one of the PDGF-responsive transcription factors but was instead modulated by transcription factor AP-1, which was associated with the ERK–MAPK signaling pathway [Fujita et al., 2008]. This evidence showed a potential link between miR-21 and ERK–MAPK signaling pathway during MSC differentiation.

Here, we found that miR-21 plays a critical role in maintaining the duration of the ERK–MAPK signaling pathway by repressing SPRY2 expression during MSC differentiation. Phosphoproteomic analysis of MSCs demonstrated that the duration and magnitude of ERK–MAPK signaling activation were significant factors in cell fate specification [Kratchmarova et al., 2005]. Only ERK–MAPK signaling was activated for a sustained period during MSC differentiation [Ng et al., 2008]. We identified SPRY2 as a desired target of the miR-21 based on its biological function. SPRY2 was not only an inhibitor of receptor tyrosine kinases but can also be induced by these kinases. The up-regulation of miR-21 could reduce the repression of the ERK–MAPK signaling pathway activity mediated by SPRY2 to sustain ERK–MAPK activation during MSC differentiation.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF MSCs

MSCs were isolated from the subcutaneous adipose tissue of a 31-year-old male. The tissue was stored in salt solution (0.9%) at 4°C before excision. The whole isolation process was performed in a sterile experimental environment. The tissue was cut into small pieces and then enzymatically digested with 0.2% collagenase type II (Sigma) for 1 h at 37°C. The digested sample was passed through a stainless steel tissue sieve and subsequently centrifuged at 1,200 rpm for 10 min. The pellet was suspended and cultured in T25 flask containing MSC growth medium at 37°C with 95% relative humidity in 5% CO_2. After 48 h, MSCs were fixed and the suspended cells were discarded. Cells were passaged when 80% confluence were reached. MSCs growth medium was composed of 58% Dubeccl's modified Eagle's medium/F12 (Invitrogen), 40% MCDB-201, 2% fetal bovine serum (FBS; Hyclone), 10 ng/ml EGF, 10 ng/ml platelet-derived growth factor (PDGF; R&D), 1 × Insulin-Tranferrin-Selenium (ITS; Sigma), 1 × linoleic acid-bovine serum albumin, LA-BSA), 50 μM β-mercaptoethanol (Merck), 2 mM L-glutamine (GibcoBRL), 100 μg/ml penicillin and 100 U/ml streptomycin. The MSCs were prepared in freezing medium consisting of 90% FBS-10% dimethyl sulfoxide (DMSO) and were stored in liquid nitrogen storage. All cells were used between passages 1 and 3.

DIFFERENTIATION

MSCs were seeded in 35-mm culture plates in growth medium according to a protocol supplied by our lab. After 24 h, the growth medium was switched to an induction medium. Half of the induction medium was refreshed every 2 days. The mesenchymal phenotype of differentiated cells was detected by staining assays (oil Red O staining and von Kossa staining) and quantitative real-time PCR assay after 14 days of induction. Osteoblastic differentiation induction medium was composed of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 μg/ml penicillin, 100 U/ml streptomycin, 10^{-7} mol/L dexamethasone (Sigma), 0.5×10^{-4} mol/L ascorbic acid (Sigma), and 10 mmol/L β-Glycerol Phosphate (Sigma). Adipogenic differentiation induction medium was composed of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 μg/ml penicillin, 100 U/ml streptomycin, 10^{-6} mol/L dexamethasone (Sigma), 7 μg/ml ascorbic acid (Sigma) and 0.5 mmol/L 3-isobutyl-*l*-methylxanthine (IBMX; Sigma). The expression of adipogenesis-associated and osteogenesis-associated genes was evaluated by quantitative real-time PCR assay from Day 1 to Day 4.

OLIGORIBONUCLEOTIDE TRANSFECTION ASSAY

Oligoribonucleotides for miRNA expression regulation were obtained from GenePharm (Shanghai, China). has-miR-21 mimics

(miR-21) was double-stranded RNA oligonucleotide that mimics the mature endogenous miR-21, and the has-miR-21 inhibitor (anti-miR-21) was designed to inhibit endogenous miR-21 expression. Both oligonucleotides have their own negative control (NC). Lipofectamine 2000 was utilized according to the manufacturer's instructions. The transfection assay was based on a previously described protocol [Luzi et al., 2008]. Transfected cells were isolated from Day 1 to Day 4 after transfection, and mRNA and protein levels were analyzed.

siRNA ASSAY

SPRY2-siRNA (hSPRY2-siRNA and scrambled SPRY2-siRNA sequences were synthesized according to published methods [Edwin and Patell, 2008] from GenePharm (Shanghai, China). Cells were suspended in MSC growth medium (GM) before transfection in 35 mm culture dishes. The transfection of MSCs with SPRY2-siRNA (200 nmol final concentration in cell culture) was performed with Lipofectamine 2000 (Invitrogen) with a standard protocol. Induction medium (IM) was changed to after transfection. RNA was isolated from MSCs cultured in adipogenic and osteogenic induction medium. qRT-PCR was utilized to detect knockdown efficiency.

QUANTITATIVE REAL-TIME PCR ASSAY

Total RNA was isolated by Trizol (Invitrogen) according to the manufacturer's instruction. The RNA concentration and purity were detected with a spectrophotometer. The isolated RNA was used for successive experiments. Approximately 500 ng of total RNA was reverse transcribed using oligo-dT primers according to the manufacturer's instructions. The cDNA was utilized as a template to amplify target genes with the SYBR Premix Ex Tag kit (TaKaRa). The primers are listed in the supplementary information (Supplementary Table 1). Each RNA sample was evaluated in triplicate. Gene expression results were analyzed with the $\Delta\Delta C_t$ method and normalized to GAPDH expression [Livak and Schmittgen, 2001]. The quantitative real-time PCR assay was performed on a Bio-Rad iQ 5 instrument. The data were analyzed using Optical System Software version 2.0.

DUAL LUCIFERASE ASSAY

The 3′-UTR sequence of PDCD4, which contains the miR-21 binding site (ATAAGCTA, NCBI RefSeq ID NM_145341.2), was amplified by PCR and cloned into the pGL3-cM dual luciferase plasmid (pGL3-cM-PDCD4), which was derived from the pGL3-control vector and constructed by Jeff Chen and Dongsun Cao in the Wang laboratory of the University of North Carolina. Either pGL3-cM-PDCD4 (500 ng) or pGL3-cM plus pRL-SV40 (50 ng) was cotransfected with miR-21 mimics (miR-21) or miR-21 inhibitors (anti-miR-21) into Hela cells in 24-well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the luciferase activity was detected by the Dual Luciferase Reporter Assay System (Promega) using a Turner Designs Luminometer Model TD-20/20 Genetic Reporter Instrument.

The 3′-UTR sequence of SPRY2, which contains the miR-21 binding site (ATAAGCTA, NCBI RefSeq ID NM_005842.2), was amplified by PCR, cloned into the pGL3-cM dual luciferase plasmid and named pGL3-cM-SPRY2 vector. MSCs were seeded in 24-well plates containing growth medium. After 24 h, the medium was change to either an adipogenic differentiation medium or an osteogenic differentiation medium. Transfection was performed using Lipofectamine^{TM} LTX and PLUS^{TM} Reagents Invitrogen based on the manufacturer's protocol. The pGL3-cM-SPRY2 (500 ng) and pGL3-cM plus pRL-SV40 (50 ng) constructs were cotransfected into the MSCs with miR-21 mimics (miR-21) or miR-21 inhibitors (anti-miR-21). Forty-eight hours after transfection, luciferase activity was detected as described above. The pRL-SV40 vector was used as a control for transfection efficiency. Each transfection condition was repeated in triplicate.

WESTERN BLOTTING

Total protein extracts were prepared in cell disruption buffer. The protein concentration was determined using a BCA standard curve. Equal amounts of protein extract were separated by SDS–PAGE gel and transferred electrophoretically to PVDF membranes (Millipore). The membranes were blocked in TBST containing 5% milk at room temperature for 1 h. After washing with TBST, the blocked membranes were probed with 1 mg/ml rabbit anti-spry2 polyclonal antibody (Millipore, 1:1,000), 200 μg/ml anti-p-ERK (E-4) mouse monoclonal antibody (Santa Cruz, sc-7383, 1:1,000), 200 μg/ml anti-ERK 1 (K-23) rabbit polyclonal antibody (Santa Cruz, sc-94, 1:1,000) or 200 μg/ml anti-α-Tubulin (TU-02) mouse monoclonal antibody (Santa Cruz, sc-8035, 1:1,000) overnight at 4°C, and subsequently washed with TBS containing 0.1% Tween 20. After these washes, the membranes were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Zhongshanjinqiao Biotechnologies, 1:5,000), for 2 h at room temperature. The secondary antibodies were detected with western chemiluminesecent reagent (Millipore). The protein level was quantified by Quantity One software.

STATISTICAL ANALYSIS

Two-tailed Student's *t*-test was used for comparing two groups (for the luciferase assay). One-way ANOVA was used for multiple-group comparisons followed by a post hoc Tukey's test (for miR-21 expression analysis during MSCs differentiation). Two-way ANOVA was used for the rest of the multiple-group comparison, followed by a Bonferroni post-test, and data were $\log_2$-transformed before analysis. All of these analyses were performed with GraphPad Prism 5.01 software.

RESULTS

ISOLATION AND IDENTIFICATION OF MESENCHYMAL STEM CELLS (MSCs) FROM ADIPOSE TISSUE

MSC are heterogeneous and lack confirming surface markers to aid in their identification. Moreover, there is a great deal variability not only among individual donors but also on different passages from the same donor [Lakshmipathy and Hart, 2008]. We isolated MSCs from a single donor to avoid results that are caused by having different donors. We first defined the isolated cells as MSCs based on their morphology, their capacity to proliferate extensively and their culture-adherent criterion in growth medium in vitro. In general, we confirmed the identity of the MSCs based on their multipotent

differentiation potential when cultured in induction medium. MSCs cultured from the same donor for <5 passages were induced to differentiate into adipocytes and osteoblasts for 14 days. Oil red O staining and van Kossa staining were used to detect adipogenesis and osteogenesis (Fig. 1A). Additionally, we performed RT-PCR assay to detect the adipogenesis-associated gene peroxisome proliferator-activated receptor γ (PPARγ), adipocyte-specific fatty acid-binding protein (ap2) and the osteogenesis-associated gene core-binding factor subunit alpha-1 (Cbfa-1), osteopontin (OPN) expression at Days 0, 7, and 14 (Fig. 1B). Both differentiation-associated genes were apparently elevation at Days 7 and 14, but they were undetectable in undifferentiated cells when cultured in growth medium (Fig. 1B). Together, we confirmed that the isolated cells from adipose tissue bear the features of MSCs in vitro. Moreover, we also detected ap2 expression during osteogenesis and OPN expression during adipogenesis. Result demonstrated that no apparent expression was detected by RT-PCR. We confirmed the adipogenic and osteogenic induction methods performed in our study were specific for each differentiation process.

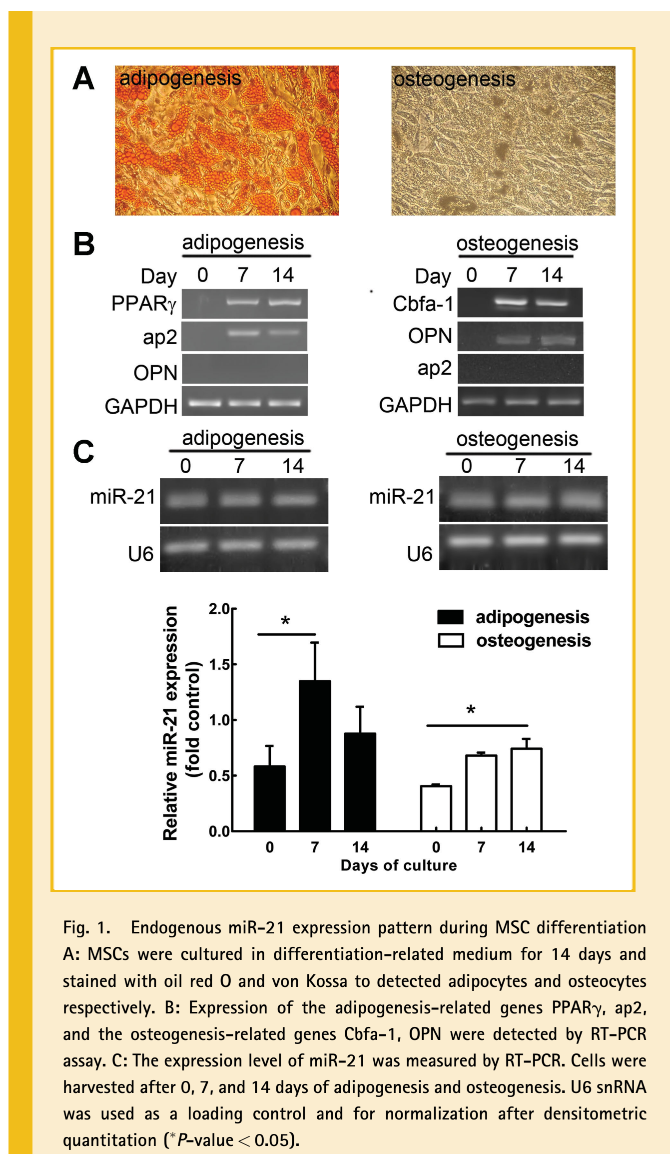

Fig. 1. Endogenous miR-21 expression pattern during MSC differentiation A: MSCs were cultured in differentiation-related medium for 14 days and stained with oil red O and von Kossa to detected adipocytes and osteocytes respectively. B: Expression of the adipogenesis-related genes PPARγ, ap2, and the osteogenesis-related genes Cbfa-1, OPN were detected by RT-PCR assay. C: The expression level of miR-21 was measured by RT-PCR. Cells were harvested after 0, 7, and 14 days of adipogenesis and osteogenesis. U6 snRNA was used as a loading control and for normalization after densitometric quantitation (*P-value < 0.05).

THE miR-21 EXPRESSION PATTERN IN MSC DIFFERENTIATION

To determine the roles of miRNAs during MSC differentiation, we studied the expression profile of miRNAs during this process by microarray (data not shown). Previous experiments showed that high levels of miR-21 were involved with many physiological and pathological processes. The miR-21 expression pattern in proliferative MSCs attracted our attention, and we sought to deduce its function in MSC differentiation. We detected the expression of miR-21 during adipogenesis and osteogenesis by RT-PCR. Analysis of miR-21 expression revealed that the miR-21 level was high when the cells were kept undifferentiated, and the expression of miR-21 remained elevated during adipogenesis and osteogenesis (Fig. 1C). Recently, two reports showed that the miR-21 expression level was elevated in MSCs during osteogenesis [Goff et al., 2008] and adipogenesis [Kim et al., 2009], but the mechanism through which miR-21 mediated these two distinct differentiation processes was unclear. Extracellular signals were identified as a fundamental element that modifies the miRNAs function by regulating their expression [Krützfeldt et al., 2006]. Meanwhile, promoter elements and transcription factors could regulate miRNA expression. Our results suggested that miR-21 was not specific for a certain differentiation process but it was likely correlate with factors and/or signal events that are commonly implicated in adipogenesis and osteogenesis.

miR-21 INCREASES THE DIFFERENTIATION POTENTIAL OF MSCs

To further explore the role of miR-21 in the differentiation of the two distinct lineages, we utilized gain-of-function and loss-of-function assay. The miR-21 mimics and its negative control were transfected into MSCs during adipogenesis and osteogenesis. In addition, we validated the specificity and the efficacy of the miR-21 mimics and the miR-21 inhibitor that were used in our experiments. We transfected a luciferase reporter into Hela cells containing a perfect complementary MRE of miR-21 (pGL3-cM-PDCD4), which provided a site with high binding affinity for the miRNA (Fig. 2Aa–c). Indeed, cotransfection of the cells with the miR-21 mimic suppressed the luciferase activity compared with the control miRNA duplexes (negative control duplex) transfected cells (Fig. 2Ad). In addition, the effect of the miR-21 inhibitor and the miR-21 mimic were opposite in the luciferase assay (Fig. 2Ae). Thus, the miR-21 mimic and miR-21 inhibitor were effective and specific for modulating the level of miR-21.

Adipogenic differentiation was induced by IBMX, Dex, and Vit C, and osteogenic differentiation was induced by Dex, Vit C, and β-GP. In this assay, we chose the first 4 days (Days 1, 2, 3, and 4) to analyze these processes. PPARγ and Cbfa-1 were indispensable transcription factors to promote adipogenesis and osteogenesis respectively. We measured the expression levels of marker genes and other differentiation-associated genes by real time PCR. In adipogenesis, an approximately twofold to threefold increase in PPARγ expression was detected by MSCs transfected with the miR-21 mimic compared with the negative control at Days 2 and 3 (Fig. 2Bb). As the downstream gene of PPARγ, ap2 level was shown an increasing tendency (Fig. 2Bf). Similar results were observed during osteogenesis. The Cbfa-1 level elevated at Days 2 and 3 and OPN level also apparently increased at Day 4 (Fig. 2Bd,h). On the contrary, these

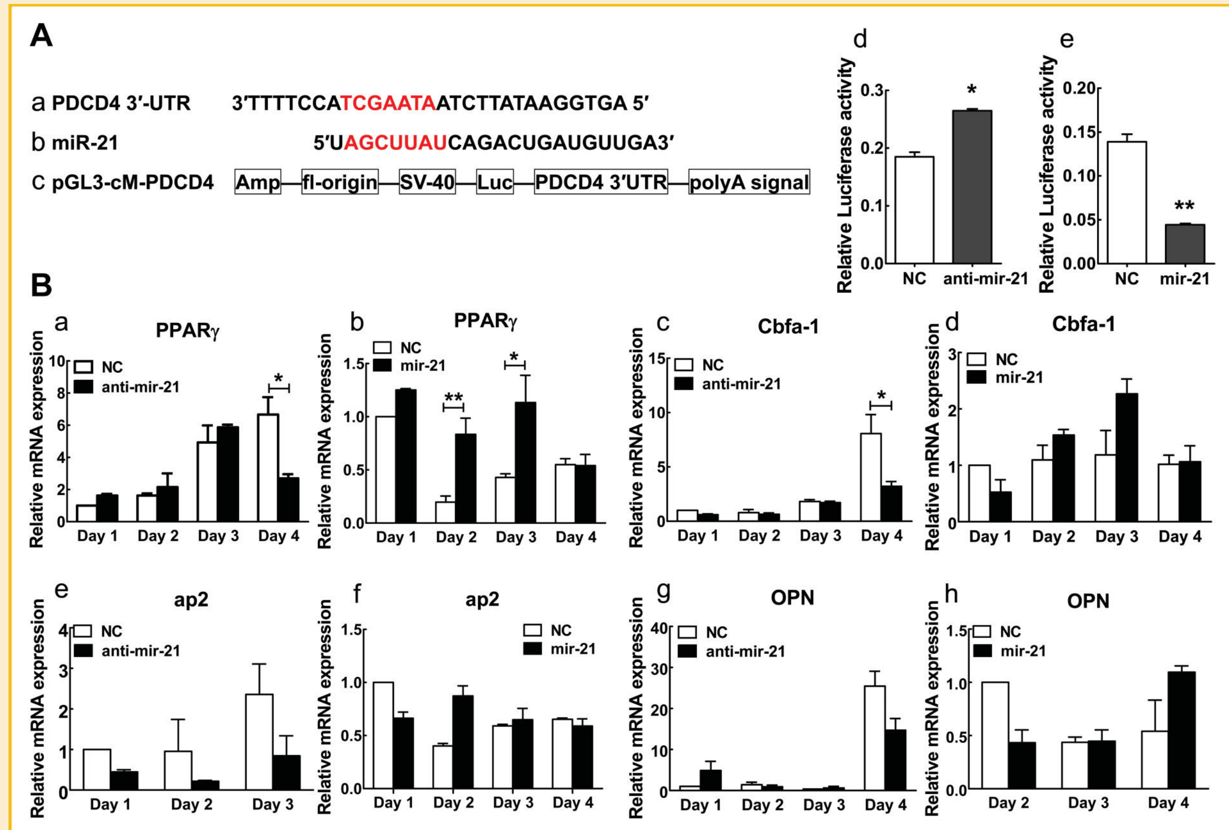

Fig. 2. miR-21 modulats MSC differentiation. A: pGL3-cM vectors were utilized to analyze miRNA activity. The restriction sites for cloning the miRNA-binding sites are located in the pGL3-cM luciferase vector. pGL3-cM cloned the part of PDCD4 3′-UTR (a), containing the miR-21 binding site (b), named pGL3-cM-PDCD4 (c). pGL3-cM-PDCD4 is co-transfected with the miR-21 inhibitor (d), the miR-21 mimic (e), or their negative control miRNAs. The pRL-SV40 vector was used as a control for transfection efficiency. Hela cells were harvested after 48 h, and luciferase activity was measured. Renilla was used to normalize transfection efficiency. B: Changes in miR-21 levels in MSCs 4 days after transfection with a negative control (NC), the miR-21 mimic (miR-21), or the miR-21 inhibitor (anti-miR-21) and the transfected cells maintained in differentiation medium (a–h). Down-regulated miR-21 expression reduced PPARγ expression compared with the NC during adipogenesis, especially at Day 4 (a). The miR-21 overexpression increased the PPARγ level compared with the NC during adipogenesis, especially at Days 2 and 3 (b). The miR-21 inhibitors can reduce Cbfa-1 expression compared with the NC during osteogenesis, especially at Day 4 (c). The miR-21 mimic increased Cbfa-1expression compared with the NC during osteogenesis, especially at Days 2 and 3 (d). As the downstream genes of PPARγ and Cbfa-1, both ap2 and OPN expression were also detected when miR-21 expression changed during adipogenesis and osteogenesis (e–h; **P*-value < 0.05, ***P*-value < 0.01).

differentiation-associated genes decreased in miR-21 inhibitor-transfected cells compared with negative control-transfected cells. The level of PPARγ sharply decreased at Day 4 during adipogenesis and ap2 level also decreased from Day 1 to Day 3 (Fig. 2Ba,e). During osteogenesis, the Cbfa-1 level decreasing occurred at Day 4 and OPN expression pattern also accorded with Cbfa-1 level changing pattern (Fig. 2Bc,g). The results indicated that the high level of miR-21 was associated with an increased differentiation potential in MSCs. However, the results of oil red O staining and von Kossa staining did not show apparent difference between the absence and presence of miR-21. These data demonstrated that miR-21 did not lead to any specific differentiation; instead it is probably involved in a common cell fate determination process, at least in the mesengenic process.

miR-21 MODULATES ERK–MAPK ACTIVITY DURING MSC DIFFERENTIATION

The ERK–MAPK signaling pathway plays key roles in MSC differentiation. Moreover, it is the only signaling pathway that is up-regulated among several differentiation-associated signaling pathways during all three MSC lineage differentiation processes [Ng et al., 2008]. The positive correlation between ERK–MAPK signaling pathway activity and the miR-21 expression pattern in differentiating MSCs was revealed during these processes. To test whether the level of miR-21 changing would affect ERK signaling activity during MSC differentiation, we detected the level of pERK by Western blot. The level of pERK was downregulated when miR-21 expression was downregulated by miR-21 inhibitors compared with the negative control during adipogenesis (Fig. 3A) and osteogenesis (Fig. 3C). The opposite results were observed when MSCs were transfected with the miR-21 mimic compared with the negative control during differentiation (Fig. 3B,D). These data indicated that ectopic expression of miR-21 promoted ERK activity during MSC differentiation. We also observed that ERK activity tended to be elevated when cells were transfected with the miR-21 negative control (NC) during adipogenesis and osteogenesis (Fig. 3B–D). The miR-21 mimic transfection could enhance this elevation (Fig. 3B,D), and the miR-21 inhibitor had an opposite effect during these processes (Fig. 3A,C). We found that the inhibition of the miR-21

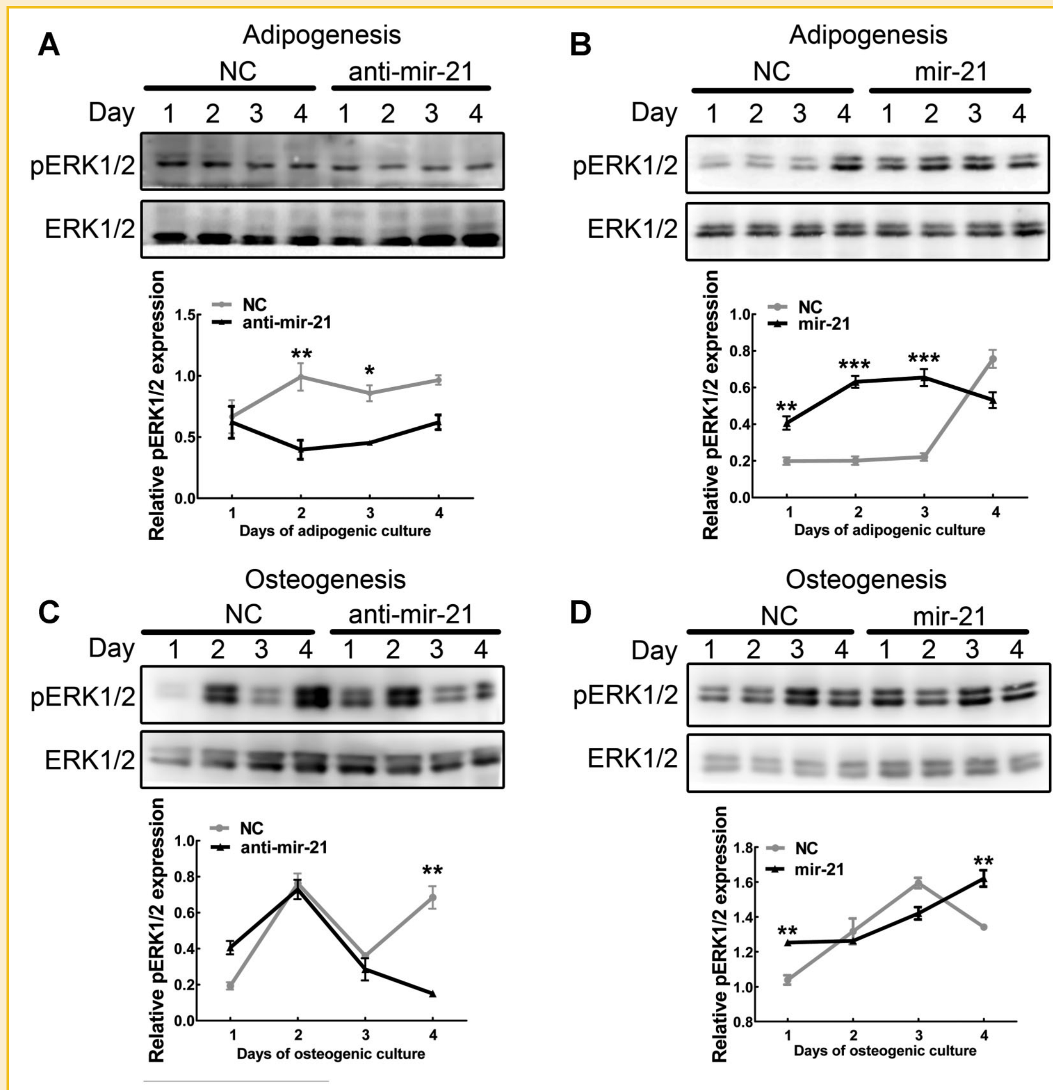

Fig. 3. miR-21 modulats ERK–MAPK signaling activity during adipogenesis and osteogenesis Western blot analysis of ERK1/2 (as a control) and phospho-ERK1/2 in differentiated MSCs. Western blots were performed on total cell lysates collected at Days 1, 2, 3, and 4 of differentiation. A,B: MSCs were transfected with the miR-21 inhibitors (A), the miR-21 mimic (B), or their negative control miRNAs at the indicated concentrations during adipogenic differentiation. C and D: MSCs were transfected with the miR-21 inhibitor (C), the miR-21 mimic (D), or their negative control miRNAs at the indicated concentrations during osteogenic differentiation. ($^{*}P$-value < 0.05, $^{**}P$-value < 0.01, $^{***}P$-value < 0.001).

inhibitor on ERK–MAPK signaling was slight, whereas the enhancement of the miR-21 mimic on this signaling was obvious. These data showed that the high level of miR-21 was essential for the ERK–MAPK signaling activity sustained during adipogenesis and osteogenesis.

TARGET GENE PREDICTION

To investigate the downstream targets underlying miR-21 regulation of MSC differentiation, we used a bioinformatics approach and summarized the context of the three widely used miRNA databases, TargetScan, PicTar, and miRanda, to identify the target genes. Bioinformatics prediction targets are likely to be categorized by specified cellular function. Most of miR-21 targets predicted by these databases are involved in ERK–MAPK pathway, and our results demonstrated that miR-21 affected the ERK activity during MSC differentiation. We hypothesized that the desired target of miR-21 should be more robustly expressed in a less-differentiated cell population than during MSC differentiation. The target is likely to interfere with the events that are essential to cell fate decision or differentiation initiation. Based on these considerations, we chose SPRY1 and SPRY2 as miR-21 target candidates. Both of genes contained the same sequence at the 3′-untranslated region (3′UTR), which is complementary to the miR-21 seed sequence (miRNA response element, MRE; Fig. 4Aa,b). SPRY1 and SPRY2 are both negative regulators of the ERK signaling pathway. To determine which candidate was a direct target of miR-21 during MSC differentiation, we detected the dynamic stage-dependent expression of SPRY1 and SPRY2. As expect, their expression patterns were

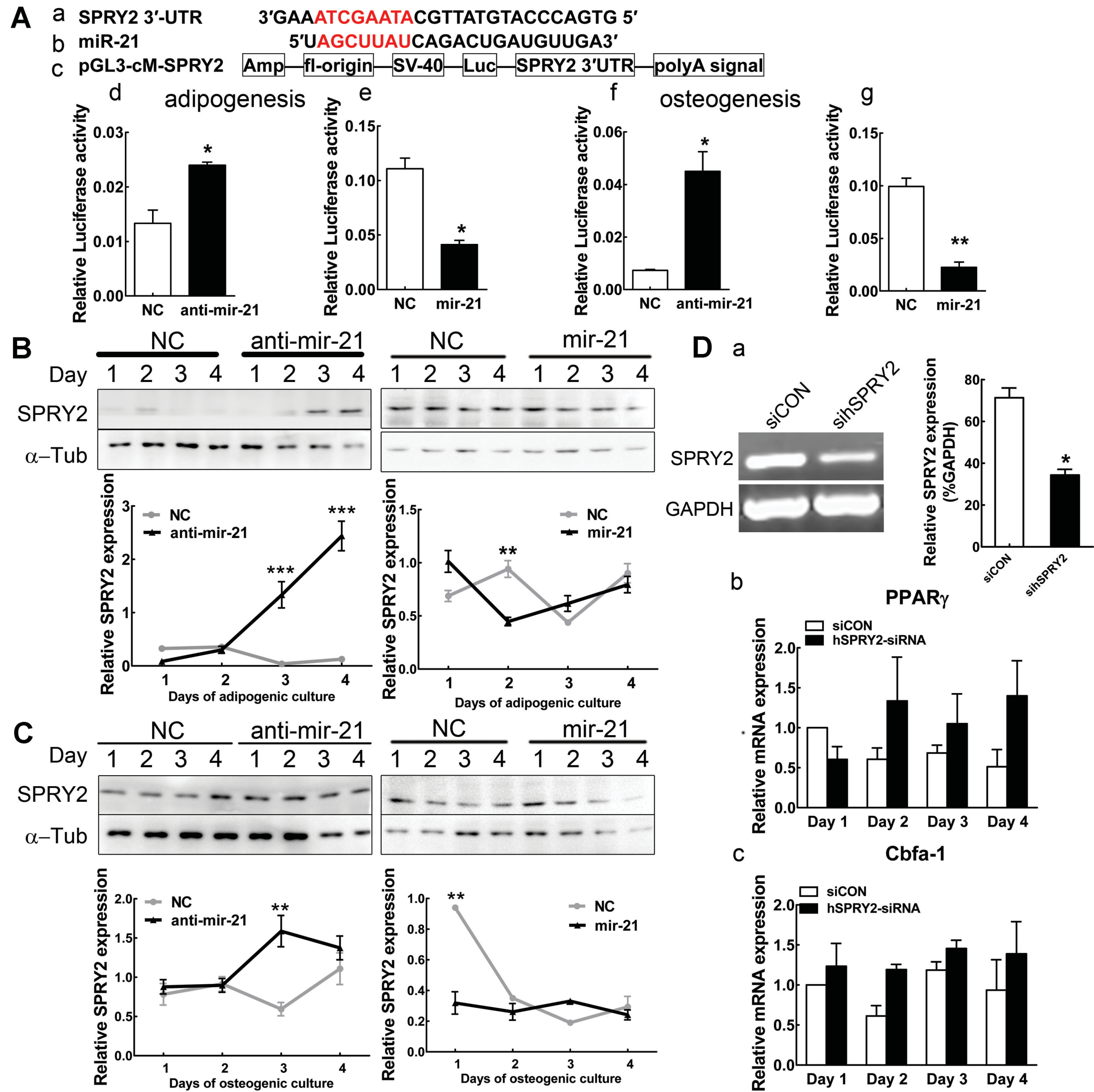

Fig. 4. SPRY2 is a target of miR-21 in adipogenesis and osteogenesis. A: The pGL3-cM vector was utilized to analyze miRNA activity. The restriction sites for cloning the miRNA-binding sites are located in the pGL3-cM luciferase vector. The pGL3-cM vector cloned the part of SPRY2 3′-UTR (a), containing the miR-21 binding site (b), named pGL3-cM-SPRY2 (c). pGL3-cM-SPRY2 is co-transfected with the miR-21 inhibitor (d), the miR-21 mimic (e), or their negative control miRNAs during adipogenic differentiation. pGL3-cM-SPRY2 is co-transfected with the miR-21 inhibitor (f), the miR-21 mimic (g), or their negative control miRNAs during osteogenic differentiation. The pRL-SV40 vector was used as a control for transfection efficiency. MSCs were harvested after 48 h, and luciferase activity was measured. Renilla was used to normalize transfection efficiency. The ratio of reporter (Firefly) to control pRL-SV40 plasmid (Renilla) was plotted in relative luminescence units. Error bars represent the standard error (n = 3). miR-21 regulates SPRY2 protein expression during adipogenic differentiation and osteogenic differentiation (B–C). B: Western blots assay for SPRY2 and α-Tubulin (as a control) were performed on total cell lysates collected at Days 1, 2, 3, and 4. B: MSCs were transfected with the miR-21 inhibitor, the miR-21 mimic, or their negative control miRNAs at the indicated concentrations during adipogenic differentiation. C: MSCs were transfected with the miR-21 inhibitor, the miR-21 mimic, or their negative control miRNAs at the indicated concentrations during osteogenic differentiation. D: Down-regulation of SPRY2 expression by transfected hSPRY2-siRNA. SPRY2 mRNA levels (normalized by GAPDH) quantified by RT-PCR (a). PPARγ and Cbfa-1 mRNA levels (normalized by GAPDH) quantified by real time PCR. Deletion of SPRY2 mRNA by transfecting MSCs with SPRY2-specific siRNA (hSPRY2-siRNA) and negative control (siCON) during adiopgenic differentiation (b). Deletion of SPRY2 mRNA by transfecting MSCs with SPRY2-specific siRNA (hSPRY2-siRNA) negative control (siCON) during osteogenic differentiation (c). (*P-value < 0.05, **P-value < 0.01, ***P-value < 0.001).

different. SPRY1 expression was undetectable by RT-PCR during proliferation (undifferentiated state), but it was strongly upregulated when differentiation was initiated. SPRY1 expression was not coincident with the temporal pattern of miR-21 expression during proliferation and differentiation. Because the binding of miR-21 to the 3′-UTR of SPRY2 has also been previously verified, we sought to determine only whether SPRY2 was a target of miR-21 using the luciferase reporter assay with differentiating MSC. We generated

directly matching miR-21 target sites and cloned these sites into the multiple cloning sites in the luciferase reporter vector pGL3-cM, named pGL3-cM-SPRY2 (Fig. 4Ac). We found that ectopic expression of the miR-21 by cotransfection of miR-21 mimic and the pGL3-cM-SPRY2 vector into MSCs suppressed the activity of a Renilla luciferase during adipogenesis (Fig. 4Ae) and osteogenesis (Fig. 4Ag). By contrast, Renilla luciferase activity enhanced when the miR-21 inhibitors and the pGL3-cM-SPRY2 vector were cotransfected in a parallel experiment (Fig. 4Ad,f). To further confirm that SPRY2 was a miR-21 downstream target during MSC differentiation, we detected the protein expression level of SPRY2 after changing miR-21 expression by transfecting the miR-21 mimic or the miR-21 inhibitor. We also found that miR-21 mimics transfected cells showed a reduction in the level of SPRY2 compared with the negative control at the same day during adipogenesis and osteogenesis (Fig. 4B,C), whereas transfecting the miR-21 inhibitor could elevate the level of SPRY2 compare with the negative control at the same day during these processes (Fig. 4B,C). We also realized that down-regulation of SPRY2 was apparent when miR-21 inhibitor transfected, but the effect of miR-21 mimic on SPRY2 expression was not apparent. We speculated that this might be because the endogenous miRNA-21 expression was too high to be affected by the miR-21 mimic. We next investigated whether SPRY2 could be responsible for the MSCs differentiation. Excessive SPRY2 expression interferes with bone development in vertebrates [Minowada et al., 1999]. Therefore, we asked whether down-regulating the level of SPRY2 would promote differentiation during adipogenesis and osteogenesis. We first determined the efficiency of hSPRY2-siRNA in transfected MSCs by RT-PCR. The SPRY2 mRNA level decreased in hSPRY2-siRNA transfected cells compared with negative control (siCON) transfected cell (Fig. 4Da). The results showed that compared with the control cells, the expression level of PPARγ was permanently upregulated from Day 2 to Day 4 when MSCs were transfected with a specific SPRY2-siRNA during adipogenesis (Fig. 4Db). In a parallel knockdown experiment, the level of Cbfa-1 also increased at Days 2 and 4 compared with the control cells during osteogenesis (Fig. 4Dc). These data support the hypothesis that SPRY2 was a target of miR-21 during adipogenesis and osteogenesis.

DISCUSSION

The molecular mechanism correlated to cell fate determination involves specific signaling pathways and certain transcription factors downstream of pathways that are sequentially activated by extracellular signals. miRNAs are indispensable modulators of regulation networks formed by signaling events and gene reprogramming during cell fate determination.

EFFECT OF THE ERK–MAPK SIGNALING PATHWAY ON MSC DIFFERENTIATION

The MAPK signaling pathway was a main determinant in initiating and maintaining the differentiation program. Therefore, the sustained administration of some MAPK signaling pathway inhibitors could convert terminally differentiated cells to a pluripotent state instead of utilizing reprogramming factors [Li and Ding, 2010]. The proteins RasGAP and extracellular-signal regulated kinase (ERK1) were commonly recognized as specific proteins that induce differentiation initiation [Xu et al., 2008]. Eliminating ERK activity was sufficient to maintain the self-renewal stage of ESCs [Chen et al., 2006]. ERK–MAPK signaling pathway was involving with mesenchymal process by regulating differentiation-associated transcription factors of these processes [Ge et al., 2007]. As lineage-specific transcription factors, PPARγ and Cbfa-1 are necessary for promoting adipogenesis and osteogenesis respectively, and both of them are associated with ERK–MAPK signaling pathway during lineage commitment. PPARγ gene appeared at Day 2 during adipogenic culture and reach maximum level at Days 3 and 4 [Ntambi and Kim, 2000]. Cbfa-1 was considered as substrates of ERK–MAPK pathway and an early cell fate marker required for osteogenic commitment [Ge et al., 2007]. Therefore, we investigated the expression of PPARγ and Cbfa-1 in absence or presence miR-21 at the early stage of induction. However, there were many controversial results on the role of ERK–MAPK pathway during adipogenesis and osteogenesis. Different methods of ERK–MAPK pathway activity alteration may result in these discrepancies, but moderate change of signaling activity was considered as optimized option and could mimic physiological process in vivo. Therefore, the precise modulation of ERK–MAPK signaling pathway is essential for MSCs differentiation. Sustained MAP kinase activity may be a prerequisite for cell fate specification. The miRNAs are endogenous modulation factors which can precisely regulate signaling transduction with time- and dosage-dependent. The miR-21 could influence this process by modulating the interaction of SPRY2 and ERK–MAPK signaling to control the duration and magnitude of the MAP kinase cascade.

SPROUTY PROTEINS AND MSC DIFFERENTIATION

In the human genome, four SPRY genes (SPRY 1–4) that encode proteins have been identified. All of the SPRY family members have a different sequence at their amino termini, which is indicative of their diverse functions. Sprouty proteins play essential roles during development by forming negative feedback loops to modulate the magnitude and duration of the ERK–MAPK signaling pathway [Casci et al., 1999]. However, due to additionally Sprouty protein functions beyond what we currently know, the subtle regulation of the ERK–MAPK signaling pathway remains unknown. Interestingly, both SPRY1 and SPRY2 contain the miR-21 binding site, which is complementary to the miR-21 seed sequence in the 3′ untranslated region (3′-UTR). The SPRY genes expression patterns were associated with mesenchymal-to-epithelial transition (MET) during development [Mason et al., 2006]. For example, SPRY seems to be specifically expressed in epithelial tissue, and expression is lost in the stroma and adipose tissue during breast development [Lo et al., 2004]. In addition, the SPYR protein level has influenced fibroblast proliferation and the duration of the MAP kinase cascade during PC12 differentiation [Gross et al., 2001]. Recently, several reports showed that SPRY family members are required for the self-renewal and differentiation of stem cells. Brack's group reported that SPRY1 expression is related to the reversible quiescence of adult muscle stem cells, but SPRY2 expression was limited in less differentiated

cells during embryonic myogenesis [Shea et al., 2010]. Importantly, SPRY2 protein elevation can inhibit bone development in vivo [Minowada et al., 1999]. Although both SPRY1 and SPRY2 can inhibit ERK–MAPK signaling activity, they play distinct roles in the differentiation process. Nevertheless, the loss of SPRY1 did not change the differentiation state: instead, it changed the self-renewal potential. SPRY1 expression was required for reversible quiescence, and the down-regulation of SPRY2 facilitated cell differentiation. Additionally, an in vivo study showed that mutant Spry2 in *Xenopus laevis* exhibited prolonged ERK activity prolonged that promoted differentiation, but the same function has not been demonstrated in the mutant Spry1 *X. laevis* [Hanafusa et al., 2009]. All of these results suggested that SPRY1 and SPRY2 play specific roles at distinct stages of differentiation. In our work, we identified SPRY2 as a bona fide target of miR-21 in agreement with previous study results [Sayed et al., 2008] and the luciferase assay performed in adipogenesis and osteogenesis as described. The duration and magnitude of MAP kinase activation was required for the regeneration process. We speculated that the miR-21 and SPRY family members interaction may fine-tunes ERK–MAPK signaling activity and gene regulation networks during MSC differentiation.

ROLE OF miR-21 DURING MSCs DIFFERENTIATION

Our results showed that the miR-21 expression level was upregulated during MSC differentiation (Fig. 1C). Previous results demonstrated that the ERK–MAPK signaling pathway played a critical role in MSC differentiation, and its activation was sustained during this process [Kratchmarova et al., 2005; Ng et al., 2008]. In this study, we found that the miR-21 expression pattern was positively correlated with ERK–MAPK activity during MSC differentiation. The miR-21 may have reduced SPRY2 level to maintain the ERK signaling activation during differentiation process. Like mRNAs, miRNA biogenesis also accorded on coding genes transcription procedure. The miR-21 promoter region has several transcription factor binding sites, including AP-1, STAT3, P53, and NF1. For example, STAT3 is a transcription factor that is regulated by the PDGF signaling pathway which is one of the most important pathways in MSC osteogenesis. However, the high expression level of miR-21 was not correlated with the PDGF signaling pathway during this process [Goff et al., 2008]. Thus, the miR-21 expression level should be regulated by another signaling event that also plays an important role in osteogenesis. Previous study revealed that the promoter region of miR-21 contains Ras-responsive elements, such as the AP-1 binding site [Fujita et al., 2008]. The high level of miR-21 may result from the duration of ERK–MAPK signaling activation during MSC differentiation. Possibly, both miR-21 and SPRY2 expression were regulated by the ERK–MAPK cascade but had opposing effects on its activation. Thus, the balance of miR-21 and SPRY2 expression could fine-tune the duration and magnitude of ERK–MAPK signaling activity to determine cell fate.

The ERK–MAPK signaling pathway is one of the components of growth signaling and may be involved in proliferation at the initiation of MSCs differentiation. Previous study showed that BMP can increase both total and phosphorylated ERK during the early phase of osteogenesis. It was implied that BMP can trigger both proliferation-associated genes and differentiation-associated genes expression during ostoeogenesis [Schindeler et al., 2006]. The proliferation process may be involved in the differentiation process, and it is impossible to distinguish such two different processes. The miR-21 promotes proliferation in several types of cancer cells. However, overexpression of miR-21 in cardiocytes was not associated with proliferation and growth [Sayed et al., 2008]. The effect of miR-21 on proliferation depends on cellular context. Previous study demonstrated that SPRY1 was abundantly expressed in most noncycling cells during muscle regeneration, and decreased in prostate cancer cells [Kwabi-Addo et al., 2004]. Down-regulation of SPRY1 expression may occur in the proliferation process mediated by ERK–MAPK signaling. On the contrary, SPRY2 mostly involved in differentiation process and overexpression of SPRY2 inhibited differentiation [Gross et al., 2007]. We also noticed that the up-regulation of miR-21 expression was accompanied with up-regulation of SPRY1 expression from undifferentiated state to initiation of MSCs differentiation. Due to different expression pattern of SPRY1 and SPRY2 during adipogenesis and osteogenesis, we speculated that they were regulated by specific modulators in response to ERK–MAPK signaling in different processes. It was possible that miR-21 and target gene SPRY2 mainly involved in the differentiation process mediated by ERK–MAPK signaling.

Feedback loop control is a major property of signaling events in response to the complex differentiation process. miRNAs and target genes interact as network motifs, participate in feedback loop network, and precisely impart intercellular signals to regulate gene expression [Tsang et al., 2007]. We speculated that miR-21 participated in the feedback loop network, including the ERK–MAPK signaling pathway and its inhibitor SPRY2. Abnormal fluctuations in the MAP kinase cascade may lead to termination of differentiation. As a modulator of this feedback loop, miR-21 may reduce such an effect and stabilize ERK–MAPK signaling pathway activation to promote differentiation. In our study, the results showed that activity of ERK–MAPK signaling pathway was more sensitive to the up-regulation of miR-21 compared with the down-regulation of miR-21 during MSC differentiation. It implies that the high level of miR-21 in MSCs is the prerequisite for the maintaining of ERK–MAPK signaling. And the function of miR-21 is modulating ERK–MAPK signaling to reach and maintain appropriate level to promote differentiation, therefore down-regulation of miR-21 is difficult to slow down the ERK–MAPK cascade. In addition, in vitro the miR-21-mediated up-regulation of ERK signaling activation seems more obvious in adipogenesis than in osteogenesis. The results suggest that the effect of miR-21 on ERK signaling activation depend on various cellular contexts. Possibly, there are other combination of modulators in the feedback loop reinforced the ERK–MAPK signaling cascade during differentiation. The mechanisms of ERK–MAPK signaling activity modulation mediated by miR-21 remain to be elucidated by further investigation.

CONCLUSION

Although adipogenesis and osteogenesis are different process during MSC differentiation, the ERK–MAPK signaling pathway is

indispensable for the intrinsic program of these differentiation processes. The ERK–MAPK pathway was defined as the differentiation-inducing signaling pathway during cell fate decisions [Ying et al., 2008]. The potential mechanism of differentiation may involve the persistent activation of intracellular transduction factors [Edlund and Jessell, 1999]. Our study revealed that the miR-21 may be not a specific noncoding RNA for adipogenesis and osteogenesis. However, the miR-21 expression up-regulation elevated the differentiation-associated transcription factors level and was associated with the ERK–MAPK signaling activation during adipogenesis and osteogenesis. The ERK–MAPK signaling pathway and its endogenous antagonist (the SPRY family members) constitute a single-negative feedback loop. We speculated that miR-21 may participate in a feedback loop regulation network that includes ERK–MAPK signaling pathway and SPRY2. Our data demonstrated that the change of ERK–MAPK signaling activity exhibited a different sensitivity to up-regulation of miR-21 and down-regulation of miR-21, and the modulation mediated by the miR-21exhibited moderate regulation for the ERK–MAPK signaling activity during adipogenesis and osteogenesis. In view of these results, multiple components involved with miR-21 in this feedback loop should be revealed and therefore we can picture a complete feedback loop regulation in the ERK–MAPK signaling pathway.

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REFERENCES

Bartel DP. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Bartel DP. 2009. MicroRNAs: Target recognition and regulatory functions. Cell 136:215–233.

Bianco P, Robey PG, Simmons PJ. 2008. Mesenchymal stem cell: Revisiting history, concepts, and assays. Cell Stem Cell 2:313–319.

Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y, Binétruy B. 2002. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem J 361(Pt 3): 621–627.

Casci T, Vinos J, Freeman M. 1999. Sprouty, an intracellular inhibitor of Ras signaling. Cell 96:655–665.

Chen S, Do JT, Zhang Q, Yao S, Yan F, Peters EC, Schöler HS, Schultz PG, Ding S. 2006. Self-renewal of embryonic stem cells by a small molecule. Proc Natl Acad Sci USA 103:17266–17271.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Edlund T, Jessell TM. 1999. Progression from extrinsic to intrinsic signaling in cell fate specification: A view from the nervous systems. Cell 96:211–224.

Edwin F, Patel1 BT. 2008. A novel role of Sprouty 2 in regulating cellular apoptosis. J Biol Chem. 283:3181–3190.

Franceschi1 RT, Xiao G. 2003. Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. J Cell Biochem 88:446–454.

Freeman M. 2000. Feedback control of intercellular signalling in development. Nature 408:313–319.

Fujita S, Ito T, Mizutani T, Minoguchi S, Yamamichi N, Sakurai K, Iba H. 2008. MiR-21 gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. J Mol Biol 378:492–504.

Ge C, Xiao G, Jiang D, Franceschi RT. 2007. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol 176:709–718.

Goff LA, Boucher S, Ricupero CL, Fenstermacher S, Swerdel M, Chase LG, Adams CC, Chesnut J, Lakshmipathy U, Hart RP. 2008. Differentiating human multipotent mesenchymal stromal cells regulate microRNAs: Prediction of microRNA regulation by PDGF during osteogenesis. Exp Hematol 36:1354–1369.

Gross I, Bassit B, Benezra M, Licht JD. 2001. Mammalian Sprouty proteins inhibit cell growth and differentiation by preventing Ras activation. J Biol Chem 276:46460–46468.

Gross I, Armant O, Benosman S, de Aguilar JL, Freund JN, Kedinger M, Licht JD, Gaiddon C, Loeffler JP. 2007. Sprouty2 inhibits BDNF-induced signaling and modulates neuronal differentiation and survival. Cell Death Diff 14: 1802–1812.

Hanafusa H, Matsumoto K, Nishida E. 2009. Regulation of ERK activity duration by Sprouty contributes to dorsoventral patterning. Nat Cell Biol 11:106–115.

Houbaviy HB, Murray MF, Sharp PA. 2003. Embryonic stem cell-specific microRNAs. Dev Cell 5:351–358.

Ivey KN, Srivastava D. 2010. MicroRNAs as regulators of differentiation and cell fate decisions. Cell Stem Cell 7:36–41.

Johnston RJ, Jr., Chang S, Etchberger JF, Ortiz CO, Hobert O. 2005. MicroRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision. Proc Natl Acad Sci USA 102:12449–12454.

Kim YJ, Hwang SJ, Bea YC, Jung JS. 2009. MiR-21 regulates adipogenic differentiation through the mofulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. Stem Cells 27:3093–3102.

Kratchmarova I, Blagoev B, Haack-Sorensen M, Kassem M, Mann M. 2005. Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 308:1472–1477.

Krützfeldt J, Poy MN, Stoffel M. 2006. Strategies to determine the biological function of microRNAs. Nat Genetics 38:S14–S16.

Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G, Ittmann M. 2004. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 64:4728–4735.

Lakshmipathy U, Hart RP. 2008. Concise review: MicroRNA expression in multipotent mesenchymal stromal cells. Stem Cells 26:356–363.

Li W, Ding S. 2010. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. Trends Pharmacol Sci 31:36–45.

Li X, Carthew RW. 2005. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell 123:1267–1277.

Liao QC, Li YL, Qin YF, Quarles LD, Xu KK, Li R, Zhou HH, Xiao ZS. 2008. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. J Cell Biochem 104:1853–1864.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408.

Lo TL, Yusoff P, Fong CW, Guo K, McCaw BJ, Phillips WA, Yang H, Wong ESM, Leong HF, Zeng Q, Putti TC, Guy GR. 2004. The Ras/Mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, Sprouty 1 and Sprouty 2 are deregulated in breast cancer. Cancer Res 64: 6127–6136.

Luzi E, Marini F, Sala SC, Tognarini I, Galli G, Brandi ML. 2008. Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. J Bone Miner Res 23:287–295.

Mason JM, Morrison1 DJ, Basson MA, Licht JD. 2006. Sprouty proteins: Multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. Trends Cell Biol 16:45–54.

Meyers VE, Zayzafoon M, Gonda SR, Gathings WE, McDonald JM. 2004. Modeled microgravity disrupts collagen I/integrin signaling during osteoblastic differentiation of human mesenchymal stem cells. J Cell Biochem 93:697–707.

Minowada G, Jarvis LA, Chi CL, Neubüser A, Sun X, Hacohen N, Krasnow MA, Martin GR. 1999. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 126:4465–4475.

Neppl RL, Wang DZ. 2009. Smooth(ing) muscle differentiation by MicroRNAs. Cell Stem Cell 5:130–132.

Ng F, Boucher S, Koh S, Sastry KSR, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V. 2008. PDGF, TGF-β, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): Transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 112:295–307.

Ntambi JM, Kim YC. 2000. Adipocyte differentiation and gene expression. J Nutr 130:3122S–3126S.

Prusty D, Park BH, Davis KE, Farmer SR. 2002. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα gene expression during the differentiation of 3T3-L1 preadipocytes. J Biol Chem 277:46226–46232.

Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. 2000. Transcriptional regulation of adipogenesis. Genes Dev 14:1293–1307.

Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, Yan L, Malhotra A, Vatner D, Abdellatif M. 2008. MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. Mol Cell Biol 19:3272–3282.

Schindeler A, David G, Little DG. 2006. Ras-MAPK signaling in osteogenic differentiation: Friend or foe? J Bone Miner Res 21:1331–1338.

Shea KL, Xiang W, LaPorta VS, Licht JD, Keller C, Basson MA, Brack AS. 2010. Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. Cell Stem Cell 6:117–129.

Singh SK, Kagalwala MN, Parker-Thornburg J, Adams H, Majumder S. 2008. REST maintains self-renewal and pluripotency of embryonic stem cells. Nature 453:223–227.

Tsang J, Zhu J, Oudenaarden AV. 2007. MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. Mol Cell 26:753–767.

Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. 2006. A micro-RNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 103:2257–2261.

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459.

Xu Y, Shi Y, Ding S. 2008. A chemical approach to stem-cell biology and regenerative medicine. Nature 453:338–344.

Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. 2008. The ground state of embryonic stem cell self-renewal. Nature 453:519–523.