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Original Article

Dicer-dependent pathway contribute to the osteogenesis mediated by regulation of Runx2

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Abstract: Osteogenesis is mediated by sophisticated interactions of various molecular functions and biological processes, including post-transcriptional regulation. A range of miRNAs have been reported to regulate bone homeostasis and osteoblasts differentiation either positively or negatively through multiple signaling pathways. RNase III endonuclease Dicer is the key enzyme required for the biogenesis of miRNAs and small interfering RNAs. To determine the global influence of miRNAs on regulation of osteogenesis of pre-osteoblast cells, the transcriptional regulation of Dicer and the function of Dicer during osteoblast differentiation and mineralization were investigated. Runx2 binding directly to the Dicer promoter region was characterized in MC3T3-E1 cells by chromatin immunoprecipitation (ChIP) and luciferase promoter reporter assays. Overexpression or knockdown of Runx2 resulted in increase or decrease of Dicer expression, respectively. Furthermore, abatement of Dicer in MC3T3-E1 cells down-regulated the expression of osteogenic marker genes and mineralization ability, at least partly involving Dicer-dependent processing of the miR-21a-5p targeting PTEN via pAKT/pGSK3β/β-catenin signaling pathways. Taken together, the study demonstrates the role of Dicer in osteogenesis and suggests that Dicer is required, in part, for Runx2 regulation of osteoblast differentiation.

Keywords: Osteogenesis, dicer, Runx2, PTEN, miR-21a-5p

Introduction

MicroRNAs (miRNAs) coded by endogenous genes are a class of single-strand, non-coding RNA molecules with the length from 20 to 25 nucleotides and participate in post-transcriptional regulation of gene expression in a variety of biological processes [1], including proliferation, apoptosis, differentiation, organogenesis and tumorigenesis [2-4]. Likewise, various miRNAs have been found to be involved in repressing their targeting genes associated with osteogenesis in bone homeostasis [5-8]. The stem-loop structures of pri-miRNA first transcribed in genes are cut into the pre-miRNA approximately in length of 60-70 nucleotides by the ribonuclease III enzyme Drosha in nucleus. Under the action of Exportin-5, the pre-miRNAs are translocated into the cytoplasm and further processed by Dicer, a second RNase III ribonuclease, into the mature miRNA and loading onto RISC complexes. Therefore Dicer is required for generation of functional miRNAs biogenesis. Hence, it's necessary to investigate the extent to which function of miRNAs are obligatory for osteogenic control of bone metabolism by examining the transcriptional regulation of Dicer and the role of Dicer in osteoblast differentiation and mineralization.

Many transcriptional factors can influence the expression of Dicer. These transcriptional factors can be cell-, tissue- or stage-specific or general. For example, the expression of the Dicer gene is positively regulated by transcription factor SOX4 through binding to promoter of dicer gene and enhancing its activity in the determination of cell fate and in the regulation of embryonic development [9]. Microphthalmia-associated transcription factor (MITF) increases the activity of the promoter of Dicer gene, thereby the expression of Dicer is stimulated

upon melanocyte differentiation [10]. On the other hand, Dicer function has been explored in skeletal development where it may be differentially expressed within the cells such as osteoblast, osteoclast, chondrocyte or neural crest cells [11-14], suggesting lineage-specific regulation on enzymatic activity of Dicer. However, there is a gap in our knowledge of the regulatory cascade from Dicer to the process of osteogenesis.

Osteogenesis is an orchestrated process requiring the physiological interplay of various factors, such as transcription factor and signaling pathway. Runt-related transcription factor 2 (Runx2), also be regarded as core-binding factor a1 (Cbfa1), osteoblast-specific factor 2 (Osf2), acts as the master regulator of bone formation. Runx2 is first expressed in early osteoblast-lineage cells and represents a lineage commitment of osteogenesis [15, 16]. Runx2 binds to the upstream region of osteogenic marker genes, such as osterix (Osx) [17], osteocalcin (Ocn) [18] and bone sialoprotein (Bsp) [19], which is described as osteoblast-specific cis-acting element (OSE). A recent study using ChIP-sequence analysis demonstrated that Runx2 could interact with chromatin across the genome, which may illustrated the transcriptional mechanisms of Runx2 contributing to the regulation of genes and pathways during osteoblast difference [20]. Our previous studies discerned a regulatory loop including Runx2, Dicer, and the miR335-5p, providing new insight into the function of Dicer in controlling differentiation of a specific cell phenotype [21]. Nevertheless, the profound influence of Dicer on osteogenesis under regulation of Runx2 is incompletely understood, but may involve the posttranscriptional mechanism by miRNAs. Despite Runx2 acts as transcription factor to start gene expression, various signaling systems are involving in the control of Runx2. For example, PTEN, a widely accepted target gene of miR21a, play a role in regulating the bone homeostasis, was proved by PTEN knockout mice with increased bone density throughout life obviously [22]. Conditional knockout of PTEN in osteoblast could accelerate fracture healing and increase the bending strength of the fracture [23]. Through inhibiting Runx2 ubiquitination, deletion of PTEN increased the amount of Runx2 post-transcriptionally [24], and PTEN inhibited the transcriptional activity of Runx2 in prostate cancer cells by inactive Akt [25]. These findings place PTEN as a key effector in the regulation of osteogenesis by Runx2.

Expression and activity of PTEN are regulated by various signaling systems and miRNAs also play a posttranscriptional regulation role. Multiple studies have reported that the miR-21a-5p directly targets PTEN, and the down-regulation of PTEN expression involves in coordinating human multipotent cardiovascular progenitors therapeutic potential [26] and modulating the immune-regulatory function of BMSC [27], but the effect of mir21a-5p/PTEN in osteogenesis was seldom reported. These results promote us to investigate the functional activity and regulation of the miR-21a-5p and target gene PTEN in osteoblast and in relation to the ribonuclease III Dicer which is required for miR-21a-5p biogenesis.

In the present study, Dicer, Runx2 and mature miR-21a-5p expressions were induced during early stage of osteogenesis and Runx2 binding in the Dicer promoter directly was identified. Further, knockdown of Dicer in osteoblast gave rise to loss of the mineralization and down-regulation of differentiation markers whereas inhibition of PTEN diminished this effect, which may be associated with related signaling. These results imply a molecular mechanism underlying lineage commitment by a regulatory network involving Runx2, Dicer and miRNAs during osteogenesis.

Materials and methods

Cell culture

The calvaria-derived preosteoblast cell line MC3T3-E1 (Subclone 4) was maintained in alpha-MEM (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (GIBCO, NY, USA) and 1 mM sodium pyruvate. Cells were grown to confluency and then changed into osteoinductive medium (10 mM β-glycerophosphate and 280 μM ascorbic acid) to induce osteogenesis. HEk-293 cells were maintained in DMEM medium supplemented with 7% FBS. Cells were maintained at 37°C in a humidified environment containing 5% CO_2 and medium was replaced every two days for the duration of all experiments.

Table 1. Sequences of sense strands of siRNA targeting Runx2, Dicer mRNA and of siRNA control

Target gene	Oligonucleotide	Sequence (5'-3')
Runx2-710	Sense	GGACUGUGGUUACCGUCAUTT
	Antisense	AUGACGGUAACCACAGUCCTT
Runx2-1263	Sense	GCCCAGGCGUAUUUCAGAUTT
	Antisense	AUCUGAAAUACGCCUGGGCTT
Runx2-1415	Sense	GCUUCUCCAACCCACGAAUTT
	Antisense	AUUCGUGGGUUGGAGAAGCTT
Dicer-1401	Sense	GCGCAAAUACAAGCCCUAUTT
	Antisense	AUAGGGCUUGUAUUUGCGCTT
Dicer-1939	Sense	GCAGAUACAGACAAGAUAATT
	Antisense	UUAUCUUGUCUGUAUCUGCTT
Dicer-3320	Sense	GGGAAAGUCUGCAGAACAATT
	Antisense	UUGUUCUGCAGACUUUCCCTT
Negative control	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT

Runx2 over-expression and Oligonucleotide transfection

MC3T3-E1 cells were plated at a density of 3×10^5 in culture plate and were transfected with pCMV-Runx2 plasmid using Lipofectamine2000 (Invitrogen, CA, USA) within 24 hs. Cells were harvested at 48 h to 72 h post transfection for RNA and protein isolation for real-time PCR and Western blot, respectively.

To assess the effect of Runx2 on mineralization, MC3T3-E1 cells were transfected with plasmid expressing Runx2 and cultured in osteoinductive medium. Media were changed every two days and Runx2 plasmid was added after 7 days.

Commercially synthesized small interfering RNA (siRNA) and miRNA mimics were transfected into the cells according to the manufacturer's protocols (GenePharma, Shanghai, CN). The siRNA targeting Dicer (siDicer) and siRNA targeting Runx2 (siRunx2) products generally consisted pools of three target-specific 19-25nt siRNAs designed to knockdown gene expression respectively. The sequences of sense strands of siRNA targeting Runx2 and Dicer mRNA and that of the negative control siRNA (siNC) are shown in **Table 1**. When cultures were approximately 80% confluent, 60 nM of siRunx2, siDicer or siNC were transfected with Lipofectamine2000.

Cells were cultured with complete medium or stimulated continuously with osteoinductive medium for indicated time after transfection and were harvested for RNA or protein isolation.

Total RNA isolation and real time PCR

Total RNA was isolated from cultures using TRIZOL reagent (Invitrogen), according to the manufacturer's protocol. For mRNAs analysis, 5 ug of total RNA was reverse transcribed the cDNA (Promega, WI, USA) in a total volume of 20 μl. A portion (0.5 μl) of the cDNA was added to a master mix containing SYBR green (Promega) and primer mixture at a concentration of 5 μM. For miRNA analysis, 2 ug of total RNA was added with polyA tail used to reverse transcript the cDNA. Real time quantitative PCR was performed using the following cycling conditions: 95°C for 10 min, 40 cycles of 15 s at 95°C and 1 min at 60°C, 95°C for 15 s, 60°C for 15 s and 95°C for 15 s (using a Bio-Rad analysis system). Expression levels were normalized to those of endogenous GAPDH or U6 and data were analyzed using the ΔΔ-Ct method. Fold change data are presented as means ± SEM. The sequence of the primers used for quantitative RT-PCR assays are shown in **Table 2**.

Western blot analysis

Western blot analyses were performed using whole cell lysates. Cells were lysed in RIPA buffer, and total protein was extracted and quantified using a BCA assay (Beyotime, Shanghai, CN). A 8% SDS-PAGE gel was used to separate 50 μg of total protein and transferred through electroblotting to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C with the primary antibody. Immunolabeling was detected using the ECL reagent. The antibodies used for western blot analysis included rabbit polyclonal anti-Dicer antibody (Abcam, MA, USA), anti-Runx2 antibody (Santa Cruz, CA, USA), anti-PTEN antibody (Santa Cruz). Anti-phospho-AKT antibody, anti-AKT antibody, anti-phospho-GSK3β (Ser9) antibody,

Table 2. Primer sequences used for quantitative RT-PCR assays

Primer of target	Sequence (5' to 3')
Dicer	Forward: 5'-CCCGAGAATTGCCTGATGGT-3'
	Reverse: 5'-TCGGTGGACCAACAATGGAG-3'
Runx2	Forward: 5'-GGGAACCAAGAAGGCACAGA-3'
	Reverse: 5'-GGATGAGGAATGCGCCCTAA-3'
ALP1	Forward: 5'-ATCGACGTGATCATGGGTGG-3'
	Reverse: 5'-TGGGAATGCTTGTGTCTGGG-3'
Osx2	Forward: 5'-ATGGCGTCCTCTCTGCTTG-3'
	Reverse: 5'-TGAAAGGTCAGCGTATGGCTT-3'
Opn2	Forward: 5'-AGAGCGGTGAGTCTAAGGAGT-3'
	Reverse: 5'-TGCCCTTTCCGTTGTTGTCC-3'
Ocn	Forward: 5'-ACCTCACAGATGCCAAGCC-3'
	Reverse: 5'-GCCGGAGTCTGTTCACTACC-3'
PTEN	Forward: 5'-TGGATTCGACTTAGACTTGACCT-3'
	Reverse: 5'-GCGGTGTCATAATGTCTCTCAG-3'
Gapdh	Forward: 5'-GGCTGCCCAGAACATCAT-3'
	Reverse: 5'-CGGACACATTGGGGGTAG-3'
β-actin	Forward: 5'-CAGCCTTCCTTCTTGGGTAT-3'
	Reverse: 5'-TGGCATAGAGGTCTTTACGG-3'
Pre-miR21	Forward: 5'-CGGATAGCTTATCAGACTGATG-3'
	Reverse: 5'-GATACCAAAATGTCAGACAGCCC-3
miR-21	5'-GCTTATCAGACTGATGTTG-3'
U6 snRNA	5'-CTTCGGCAGCACATATACTAAAATT-3'

anti-GSK3β antibody, anti-β-catenin antibody and anti-β-actin antibody were purchased from Cell Signaling Technology (CST, MA, USA).

Immunofluorescence assay

MC3T3-E1s were seeded onto coverslips in 24-well plates at a density of 2 × 10^4 cells/well and cultured to 80% confluence. The cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA) for 15 min, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Next, the cells were blocked, and then incubated with primary antibodies overnight at 4°C. For immunofluorescence analysis of Runx2 and Dicer, red-fluorescent Cy3-labeled goat anti-rabbit IgG antibody (Beyotime) was used at a dilution of 1:100. Nuclear staining was performed with DAPI and localization of Runx2 and Dicer was visualized by confocal microscope.

Identification of a putative Runx2 binding site in the dicer promoter and plasmid construction

To identify potential binding sites for Runx2 in the Dicer promoter, the JASPAR CORE database (http://jaspar.genereg.net/) was used to screen the promoter region of Dicer from -2000 to +1 bp of the translation start site. Our analysis identified three putative Runx2 binding sites (TGTGGT) at positions -1424 bp, -1357 bp and -464 bp upstream of the Dicer translation start site.

To generate the pGL3-Dicer promoter construct, a 1049 bp fragment of Dicer promoter ranging from -420 to -1469 bp was PCR amplified from mouse genomic DNA using High Fidelity polymerase (takara, Tokyo, Japan) with following forward primer 5'-GTATAGCTAGCATATATGTATGTGCCAGGGCTTTG-3' and reverse primer 5'-ACCAAGCTTTTGGTCCAAAGAACTGATCTCTT-3'. The fragment was cloned directionally between Hind III and Xho I sites of pGL3 basic vector (Promega) and the construct was verified by sequencing.

Luciferase assay

Hek293T cells were co-transfected with pCMV-Runx2 plasmid of gradual increase dose from 100 ng to 400 ng and pGL3-Dicer promoter constructs using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). MC3-T3-E1 cells were co-transfected with pGL3-Dicer promoter plasmid mixed with siRunx2 or pCMV-Runx2 with lipofectamine2000. As an internal control, the cells were co-transfected with the pRL-TK plasmid expressing Renilla luciferase, and the firefly luciferase signal was normalized to that of Renilla. After 48 hs post transfection, cells were lysed in 1 × passive lysis buffer and collected. Dual-luciferase system (Promega) was used to measure the promoter activity. Luminescence was detected using a Single-Tube Multimode Reader (Glo-Max®-Multi Jr, Promega). The data represent mean ± SD of three experiments performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay

MC3T3-E1 cells were treated with or without osteoinductive medium for 72 hs. ChIP was performed with a SimpleChIP™ Enzymatic Chromatin IP Kit (Magnetic Beads) (CST, MA, USA) according to the manufacturer's instructions. To crosslink proteins to DNA, add 540 ul of 37% formaldehyde to each 15-cm culture

dish containing 20 ml medium for 10 minutes, and the reaction was then stopped through the addition of glycine at a final concentration of 0.125 M. Add 1.5 ul of micrococcal Nuclease for 20 min at 37°C with frequent mixing to digest DNA and stop digest by adding 100 ul of 0.5 M EDTA. The cell lysate then was sonicated to obtain DNA fragments of 200 to 1000 bp. The anti-Runx2 antibody was used to immunoprecipitate the DNA-protein complex, and IgG alone was used as a negative control. After overnight incubation with the antibody, immunocomplexes were collected using 30 μl of CHIP Grade Protein G Magnetic beads and incubate for 2 h at 4°C. The magnetic beads were washed with the following buffers: low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer. DNA was eluted with elution buffer and subjected to reverse cross-linking, proteinase digestion, and purification using a spin columns. The primers used to amplify the mouse Dicer promoter region are 5'-aaagacagccgaggatgtgg-3' and 5'-aagatccaactgcctctgcc-3', and primers used to amplify osteocalcin promoter region are 5'-CTAATTGGGGGTCATGTGCT-3' and 5'-CCAGCTGAGGCTGAGAGAGA-3'. The results from realtime-PCR were used to calculate the IP efficiency manually using Percent Input Method. With the method, signal obtained from each immunoprecipitation are expressed as a percent of the total input chromatin. The percentage of chromatinimmune complex precipitate DNA relative to input was calculated and shown as mean ± SE for three independent experiments.

Proliferation assay

MC3T3-E1 cells were seeded in 96 well plates (5×10^3 cells/well) and transfected with siDicer or siNC. Cells were cultured for 7 days and the proliferation was assessed by Cell Counting Kit-8 assay system (Beyotime) at 24 h intervals. According to the manufacturer's instructions, cells were incubated with 10 μl of CCK8 reagent for 2 hs at 37°C and the optical density was measured at 450 nm using Microplate Reader.

ALP activity

MC3T3-E1 cells were seeded in 6 well plates (5×10^4 cells/well) and transfected with or without siDicer. Cells were cultured in osteoinductive medium for 8 days. The cells and medium supernatants were collected every other day, so the values represented production or secretion of the ALP over two days.

For ALP activity analysis, the cells were lysed into 1% TritonX-100 and homogenized by sonication for 20 s. The ALP activity of cell lysates and supernatant were measured following the instructions provided with the ALP kit (Jiancheng, Nanjing, CN). Activity of cell lysate was normalized to total cellular protein by Bradford protein assay.

ALP and ARS stain

MC3T3-E1 cells were seeded in 6 well plates (5×10^4 cells/well) and transfected with si-Dicer. Cells were also cultured in growth media without the Dicer knockdown as a control. Mineralization was induced by culturing cells in osteoinductive medium.

After 7 days, the level of ALP staining was determined using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime). According to the manufacturer's instructions, the cells were rinsed three times with PBS and then treated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution for 30 min.

After 14 days, cells were fixed with 4% paraformaldehyde, washed three times with PBS and then stained with 0.5% alizarin red-S (pH 4.2) for 30 min at room temperature. Free calcium ions were removed by washing with PBS three times. The stained cultures were then photographed.

Statistical analysis

All experiments were performed at least three times. Results are expressed as the mean ± SEM of duplicate independent samples. Unpaired Student's t-test was used to determine the statistical significance of difference between two groups. Analyses were conducted using a SPSS 19.0 software. $P < 0.05$ was considered to be statistically significant.

Results

Dicer protein and mRNA levels are upregulated coincidence with the expression of Runx2 during osteogenesis

Immunofluorescence imaging by laser confocal microscopy showed that the expression of Dicer and Runx2 in MC3T3-E1 cells during osteogenesis was more than that in control cells, and Dicer mainly located in cytoplasm while Runx2 predominantly expressed in nucleus (**Figure 1A**).

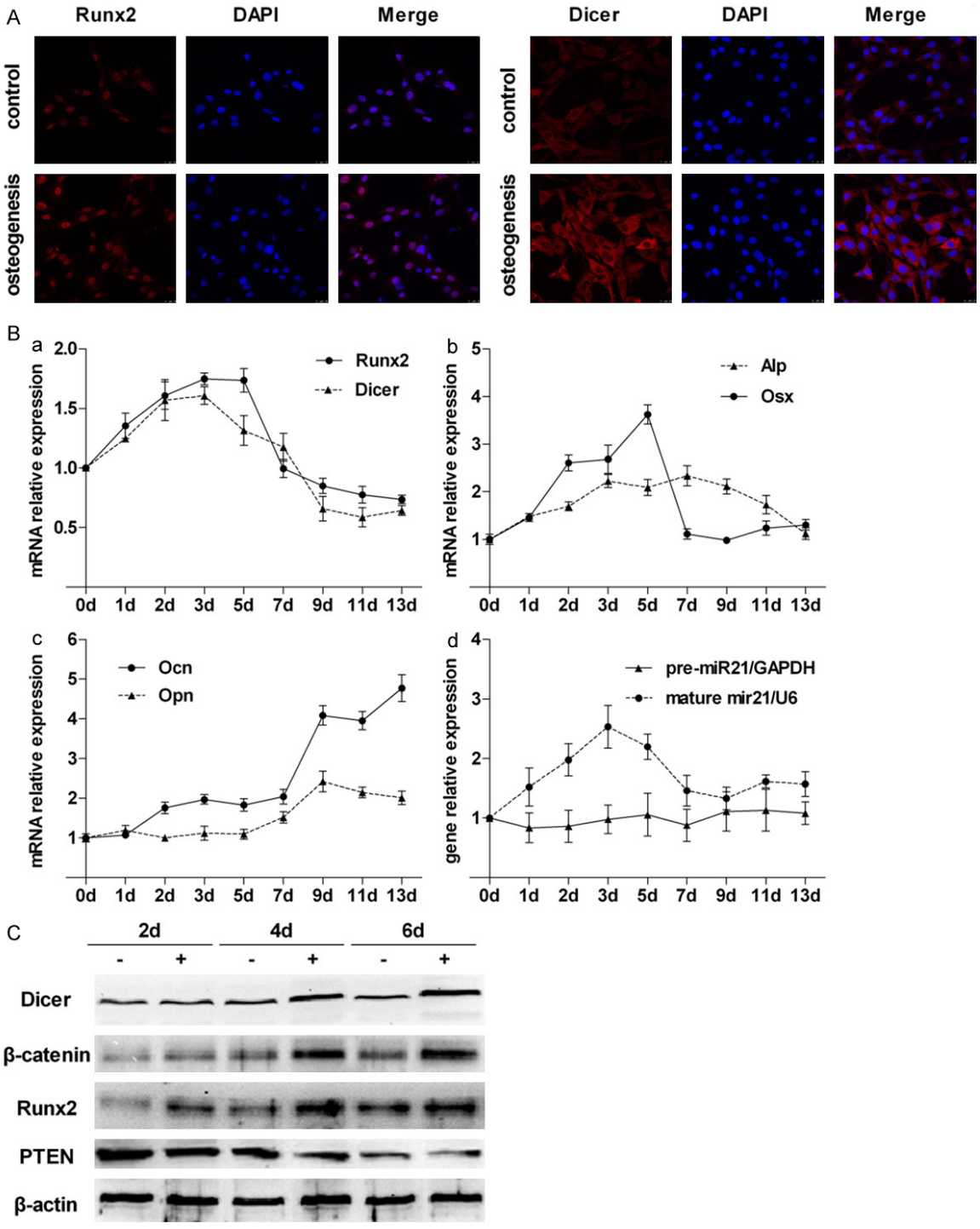

Figure 1. Expression of Runx2 and Dicer during osteogenesis. (A) Immunofluorescence analysis to assess the expression of Runx2 and Dicer in MC3T3-E1 cells during osteogenesis (lower panel) in comparison to control cells (upper panel). Scale bar = 20 μm. (B) mRNA Expression profiles of Runx2, Dicer (a), ALP, Osx (b), Opn, Ocn (c) and pre-miR21, miR21a-5p (d) were detected by real-time RT-PCR. Relative mRNA and pre-miRNAs expressions are normalized to GAPDH and mature miRNAs to U6 and are presented relative to the control cells at day 0. These data are expressed as the mean ± SEM (n = 3). (C) Protein levels of Dicer, β-catenin, Runx2 and PTEN in MC3T3-E1 cells during osteogenesis were determined by western-blot, β-actin was used as loading control.

To assess expression changes in osteogenic marker genes during the differentiation from pre-osteoblast to mature osteoblast cells, a time course of mRNA and miRNA expression

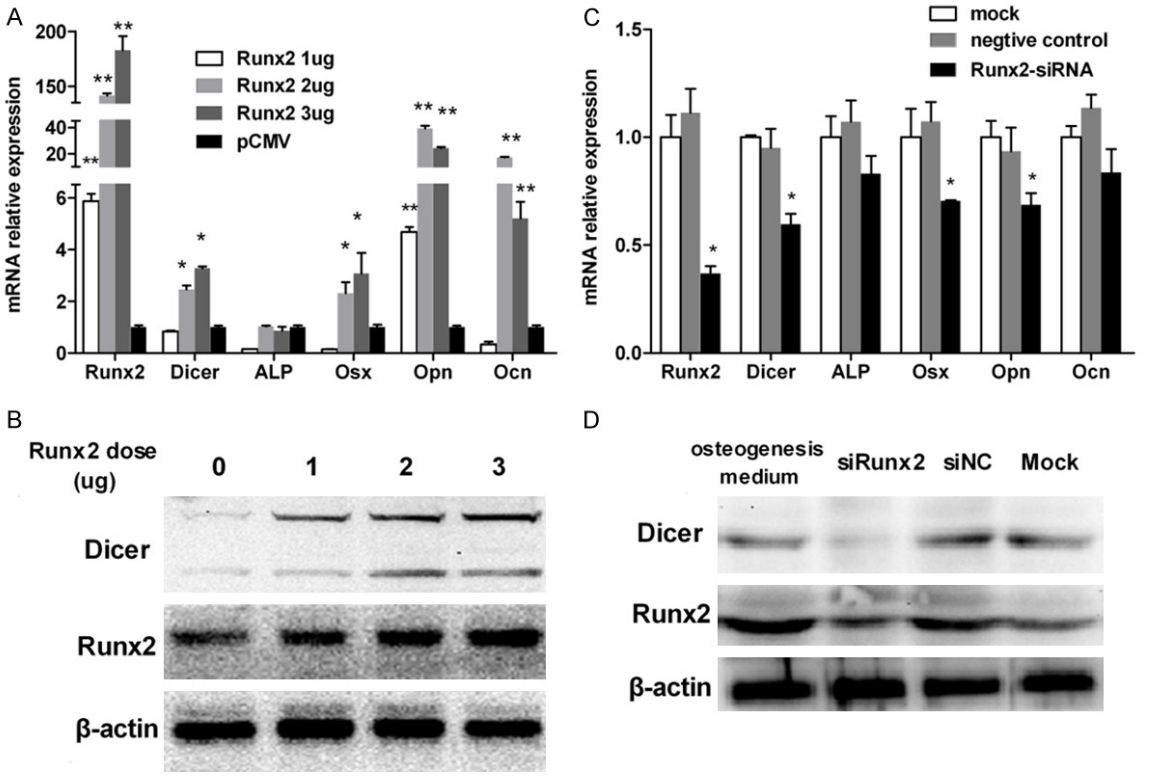

Figure 2. Runx2 up-regulates Dicer mRNA and protein expression. A. MC3T3-E1 cells transiently transfected with indicated amounts of pCMV-Runx2 plasmid and cultured for 48 h. The mRNA expression levels of Runx2, Dicer, ALP, Osx, OCN and OPN were measured by real-time PCR. Results are normalized to GAPDH and are relative to pCMV vector group values. B. MC3T3-E1 cells treated the same as above were cultured for 72 h. Protein levels of Dicer and Runx2 were detected by Western blot analysis using β-actin as loading control. C. MC3T3-E1 cells were transfected with siRunx2, siNC or Mock and cultured for 48 h. The mRNA expression levels of Runx2, Dicer, ALP, OSX, OCN and OPN were measured by real-time PCR. Results are normalized to GAPDH and are relative to Mock group values. D. MC3T3-E1 cells were treated the same as above and cultured for 72 h. Protein levels of Dicer and Runx2 were detected by Western blot analysis using β-actin as loading control. A, C. These data are expressed as the mean ± SEM (n = 3). *indicates significant differences between the indicated groups. *P < 0.05, **P < 0.01. B, D. The images represent the result of three replicates.

of MC3T3-E1 cells inducted to osteogenesis from 0 to 13 day were performed. Runx2 increased as cells proceeded from proliferation (day 0) to day 3 and decreased upon day 5 and a similar trend was observed in Dicer but decreased more earlier on day 3 (**Figure 1Ba**). Alkaline phosphatase (ALP), an early osteoblast marker, mRNA increased steadily from day 0 to day 7 and began to declined gently. Osterix (Osx/sp7), a TF of committed osteoprogenitors, significantly increased during the initial stage of differentiation upon day 5 and rapidly declined on day 7 (**Figure 1Bb**). An initial slight increase was found in the specialized mineral binding proteins secreted phosphoprotein 1 (OPN/Spp1) and osteocalcin/bone gamma-carboxyglutamate (gla) protein 2 (OCN/Bglap2). While the OCN mRNA levels reached steady state, the OPN mRNA expression declined during late mineralization on Day 9 (**Figure 1Bc**). As shown in **Figure 1Bd**, pre-miRNA21 remained at relatively steady level, a trend was observed for the increase in mature miR21a-5p, suggested posttranscriptional regulation of the miRNA. The expression profile displayed expression patterns consistent with the progression of osteogenesis and suggested a relationship between Runx2, Dicer and miR21a-5p to regulate the osteoblast differentiation at multiple stages.

Cells were cultured in osteoinductive medium for 2, 4 and 6 days respectively and then detected by western-blot, showing that osteoinductive medium significantly induced Dicer, β-catenin and Runx2 protein production in a time-dependent manner, but reduced PTEN expression (**Figure 1C**).

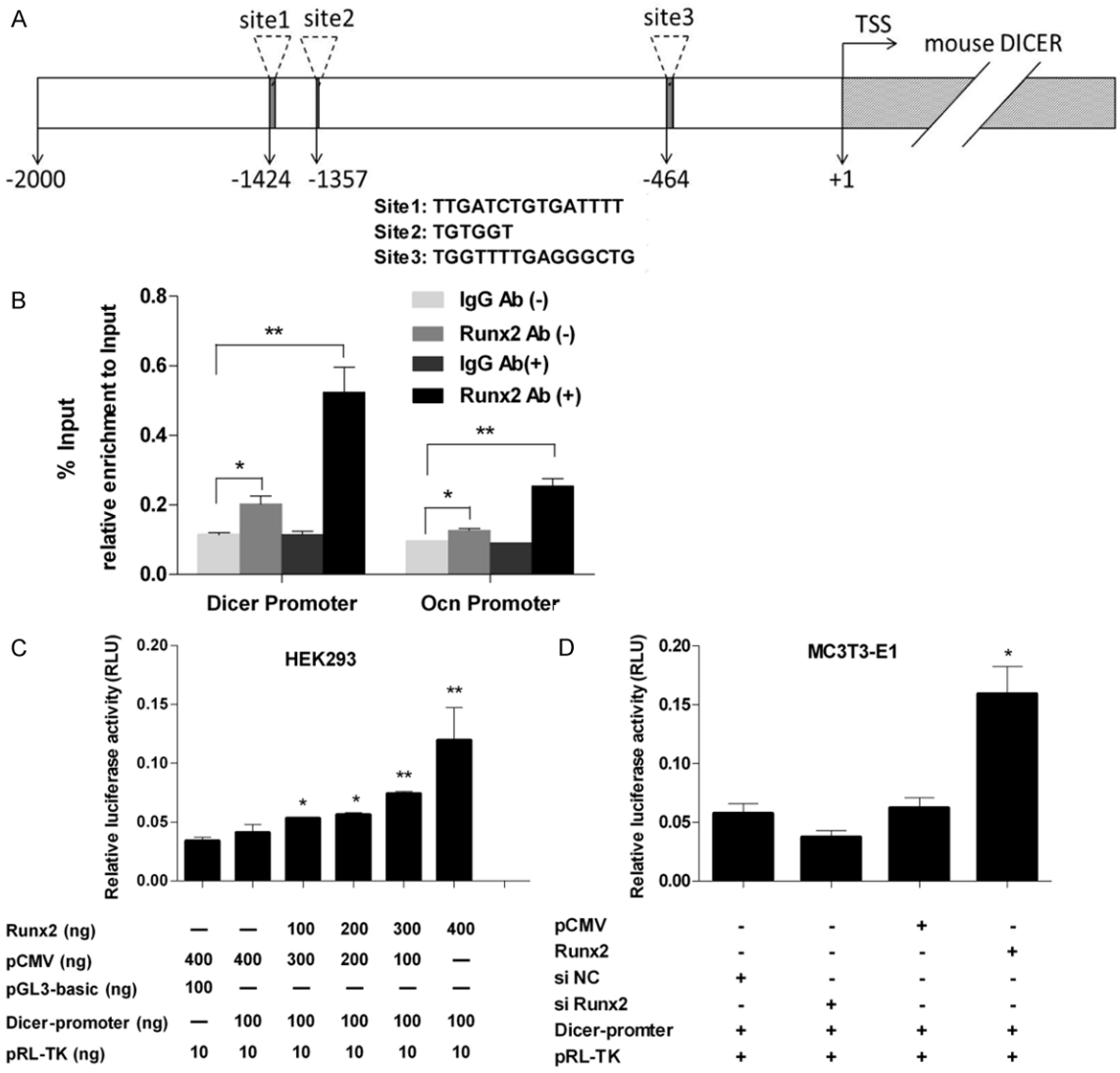

Figure 3. Runx2 Direct Regulation on the Dicer Promoter. A. Schematic diagram of the mouse Dicer gene promoter showing the positions of potential binding sites of putative transcription factor Runx2 analyzed using Jarsper. Three segments at -1424 bp (site1), -1357 bp (site2) and -464 bp (site3) upstream from the transcription start site (TSS). B. Binding of Runx2 to the mouse Dicer promoter region in preosteoblast cultured with osteoinductive medium or control (-). Chromatin immunoprecipitations were performed using log-phase MC3T3-E1 cells. Protein-chromatin-crosslinked complexes were immunoprecipitated with either Runx2 antibody or negative control antibody IgG. Chromatin immunoprecipitate obtained in the ChIP assay was quantified by real-time PCR. PCR primers spanning DICER promoter including the three putative binding sites regions were employed. OCN promoter primers were used as a positive control. The data show promoter occupancy normalized to Input and represent Mean ± SEM of three independent experiments, *P < 0.05. C. Activity of the Dicer promoter in HEK293 cells co-transfected with the indicated amounts of pCMV-Runx2 plasmid, pGL3-dicer promoter and pRL-TK. Dose dependent increase of Dicer promoter activity in HEK293 cells with increasing concentration of pCMV-Runx2 plasmid was measured by dual-luciferase assay. Luciferase values expressed were normalized with respect to pRL-TK value. D. MC3T3-E1 cells were transfected with pCMV vector, pCMV-Runx2, siNC or siRunx2 respectively and induced for 48 h. Total cell lysates obtained from these cells were assayed for luciferase activity and normalized with pRL-TK activity co-expressed in these cells. C, D. Data represent triplicate studies with a mean ± SEM. *P < 0.05, **P < 0.01.

Runx2 positively regulates expression of dicer in MC3T3-E1

To explore the possibility of Runx2 up-regulation on Dicer, MC3T3-E1 cells were transiently transfected with 1, 2, 3 ug of pCMV-Runx2 plasmid or pCMV vector respectively. The varying concentration of Runx2 in the transfected MC3T3-E1 cells were confirmed and the Dicer mRNA expression correspondingly increased

0.84, 2.45 and 3.27 fold respectively (**Figure 2A** and **2B**), suggesting the endogenous Dicer mRNA and protein levels increasing in a Runx2 dose dependent manner. A similar dose dependent was observed in Osx, a reported downstream gene regulated by Runx2. Over-expression Runx2 also significantly increased OPN and OCN mRNA expression, whereas had no effect on expression of the gene encoding ALP, an enzyme present on the surface of mineralizing cells and their derived matrix vesicles that promotes hydroxyapatite crystal growth.

As a complementary approach, cells were also transfected with siRunx2, siNC prior to osteogenic induction. These siRNA have been shown to efficiently knockdown Runx2 mRNA and protein levels. Moreover, MC3T3-E1 transfected with siRunx2 showed significantly lower levels of endogenous Dicer (**Figure 2C**, **2D**) and downregulation of other osteogenic marker gene was seen to be affected by Runx2 activity.

Runx2 directly binds to the dicer promoter

Examining the promoter of Dicer gene in mouse revealed 3 cis-acting elements OSE, the Runx2 consensus binding sequences, within -2 kb upstream of the Dicer transcription start site (**Figure 3A**).

The binding of Runx2 to the Dicer gene promoter region was detected by ChIP assay using anti-Runx2 antibody as described. Chromatin immune complexes obtained from osteoinductive medium cultured MC3T3-E1 cells upon 3 days were analyzed by qPCR using Dicer promoter gene specific primers for the Runx2-binding region. As shown in **Figure 3B**, Osteogenic induction significantly increased Runx2 binding to the Dicer promoter region, while less binding on the promoter in Chromatin immunoprecipitation obtained with a control IgG or from cells in non-induced (-). A similar enrichment of Runx2 binding was detected in the promoter region of osteocalcin, a gene whose transcription is known to be regulated by Runx2, and served as a positive control (**Figure 3B**).

To study the transcriptional activity of Runx2 on Dicer promoter, transient co-transfection experiments were performed on HEK-293 cells and demonstrated a dose dependent increase in Dicer expression, with increasing concentrations of transfected pCMV-Runx2 plasmid (**Figure 3C**). To determine the potential role of Runx2 in modulate Dicer gene promoter activity under osteogenic stimulation, MC3T3-E1 cells were transiently co-transfected with pGL-Dicer promoter reporter plasmid with siRunx2, siNC, pCMV-Runx2 or empty vector pCMV and then cultured in the osteoindctive medium for 48 h. Over-expression of Runx2 promoted the transcription of Dicer, while siRunx2 inhibited Dicer promoter luciferase reporter gene activity. In contrast, no significant change in gene promoter activity was observed in cells transfected with empty vector and siNC (**Figure 3D**). These results indicated that Runx2 could occupy and directly regulate the endogenous Dicer promoter.

Dicer knockdown in preosteoblast leads to reduced osteogenesis

MC3T3-E1 cells were transfected with siRNA targeting Dicer and cultured in complete medium or osteoinductive medium separately. As the growth curves shown, knockdown of Dicer promoted the proliferation of MC3T3-E1 (**Figure 4A**). The osteogenic phenotype of Dicer knockdown cells was assessed by detecting the enzymatic activity and calcification of ALP as well as the expression levels of several osteoblastic genes. Dicer knockdown significantly inhibited the induced ALP activities of cell lysate and cultural supernatants (**Figure 4B**). Staining for ALP calcification and Alizarin red for mineral deposition (**Figure 4D**) revealed the Dicer knockdown significantly inhibited osteoblast maturation while Runx2 overexpression promoted ALP expression at day 7 and mineralization at day 14. MC3T3-E1 cells in Dicer knockdown or control were both cultured in osteoinductive medium and assessed at 24 h intervals from day 0, when the expression of these genes was initiated, until day 5 of induction and **Figure 4C** showed that Dicer knockdown could decreased the expression of osteogenic genes, such as Runx2, ALP, OSX, OCN and OPN, whereas expressed significantly greater amounts of PTEN. To explore the biological function of Dicer enzyme in osteogenesis, we examined potential functional protein expression by western-blot in cells transfected with siDicer and cultured in osteoinductive medium for 2, 4, 6 days. Decreased protein levels of β-catenin and Runx2 in response to Dicer knockdown and a tendency towards of higher PTEN protein were also observed in **Figure 4E**.

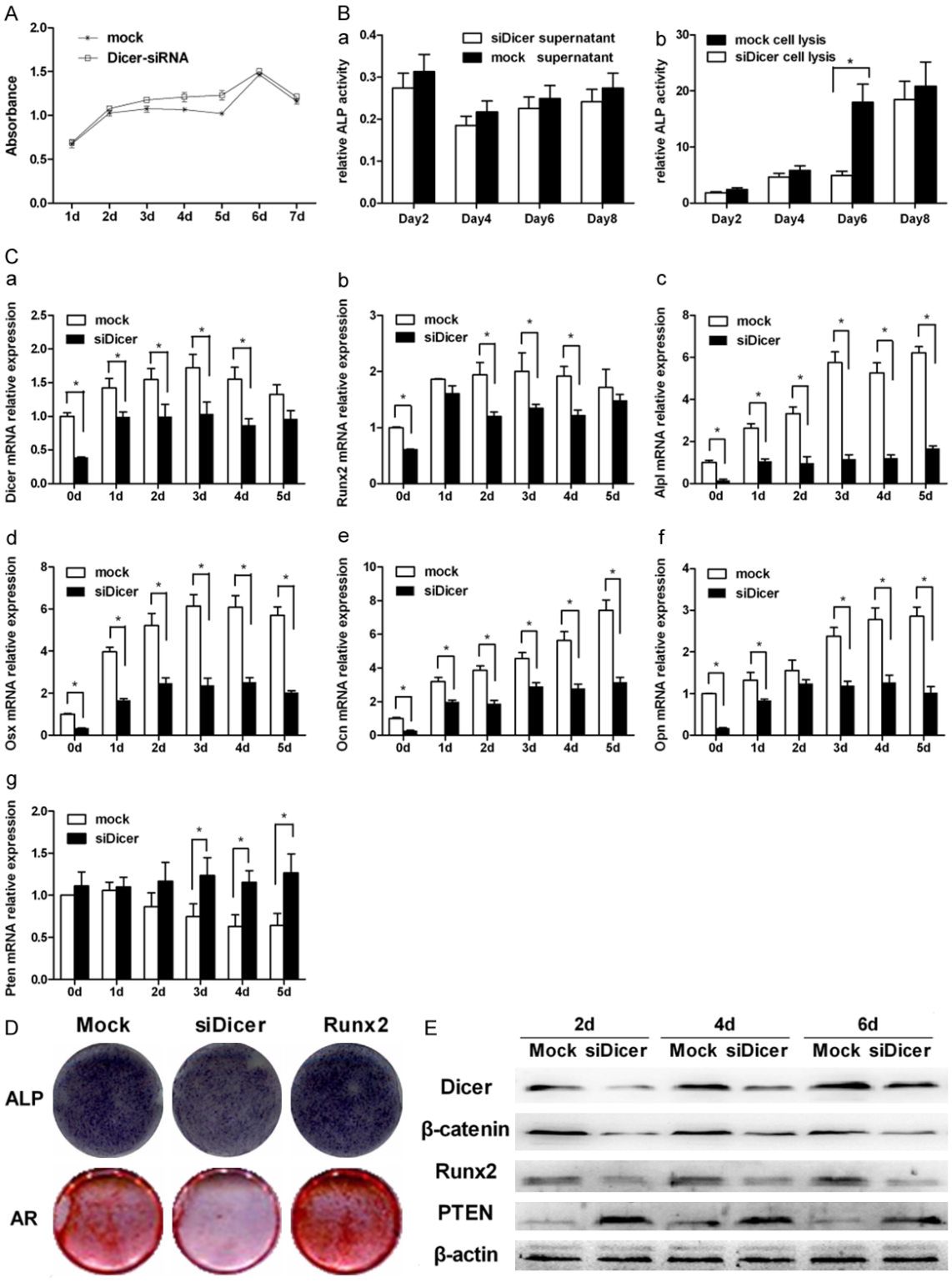

Figure 4. Dicer knockdown attenuates the osteogenesis of MC3T3-E1. (A) CCK8 proliferation assay for cells transfected with siNC or siDicer at different time, (n = 3). (B) Cells transfected with or without siDicer were cultured in osteoinductive medium. ALP activity in MC3T3-E1 supernatant (a) and cells lysis (b) were quantitative reading by spectrometer and normalized with protein contents. Data represent triplicate studies with a mean ± SEM, *P < 0.05. (C) Osteogenic marker genes expression determined by real-time RT-PCR during osteogenesis of Dicer knockdown cells. Relative mRNA expressions were normalized to GAPDH and relative to the control cells at day 0 not exposed to

osteoinductive medium. The data are represented as the mean ± SEM of three experiments. *indicates significant differences between the two groups, *P < 0.05. (D) MC3T3-E1 cells transfected with siDicer, pCMV-Runx2 and mock respectively were cultured in osteoinductive medium. ALP activity was detected by BCIP/NBT staining after induced for 7 days (upper, panel) and mineralization was determined by Alizarin red stain after induced for 14 days (lower, panel). (E) MC3T3-E1 cells were treated the same as above, and protein was prepared for Western blot analysis at 2, 4, 6 days using anti-Dicer, anti-Runx2, anti-PTEN, anti-β catenin and anti-β-actin antibodies.

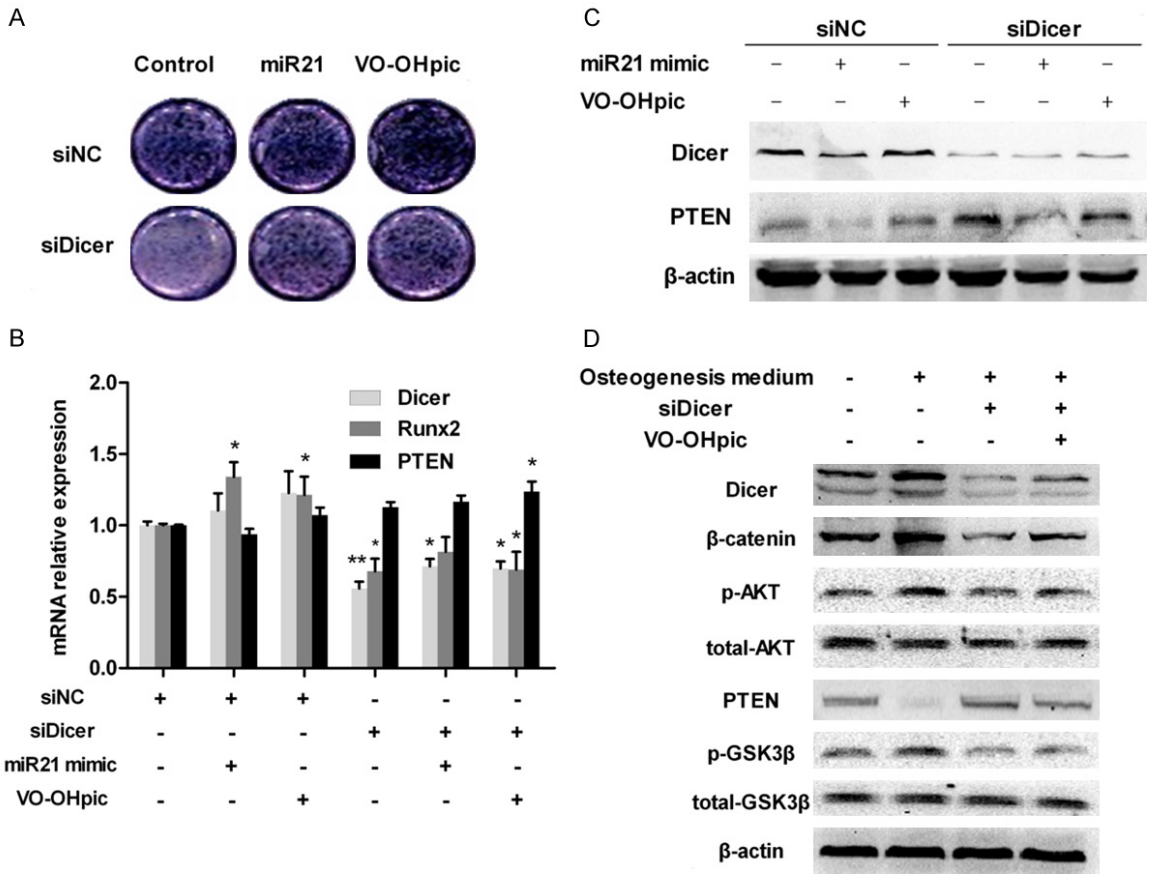

Figure 5. Regulation of miR-21a-5p and PTEN by Dicer during osteogenesis. A. After treating with siDicer or siNC for 24 h, MC3T3-E1 cells were transfected with miR-21a-5p mimics or blocked with PTEN by inhibitor VO-OHpic trihydrate. Cultured in osteoinductive medium for 7 days, the cells were fixed and stained with BCIP/NBT. B. MC3T3-E1 cells were treated the same as above and cultured in osteoinductive medium for 48 h. The mRNA expression levels of Dicer, Runx2 and PTEN were measured by real-time PCR. Results are normalized to GAPDH and are relative to negative control siRNA group values. These data are expressed as the mean ± SEM (n = 3). *indicates significant differences between the indicated groups (P < 0.05). C. MC3T3-E1 cells were treated the same as above and cultured in osteoinductive medium for 72 h. Protein levels of Dicer, Runx2 and PTEN were detected by Western blot analysis using β-actin as loading control. D. PTEN regulates osteogenesis through AKT/GSK3β/β-catenin pathway signaling in Dicer knockdown cells. MC3T3-E1 cells transfected with siDicer or blocked with PTEN inhibitor VO-OHpic trihydrate were cultured in the presence or absence of osteoinductive medium for 24 h. Protein levels of Dicer, β-catenin, total and phosphorylated AKT, PTEN, total and phosphorylated GSK3β on Ser9 were detected by Western blot analysis using β-actin as loading control.

miR-21a-5p regulates β-catenin by targeting PTEN involving Akt/GSK3β signal pathway

From the mRNA and miRNA expression profile of osteogenic induced MC3T3-E1 cells, we found that miR-21a-5p was upregulated in differentiated osteoblast (**Figure 4Cd**) correlated with PTEN protein reduction (**Figure 4Cg**), suggesting miR-21a-5p which may functionally contribute to this osteogenesis phenotype. ALP staining demonstrated that Dicer Knockdown resulted in significant reduction of ALP production, while restoration of miR-21a-5p or inhibition of PTEN with VO-OHpic trihydrate in

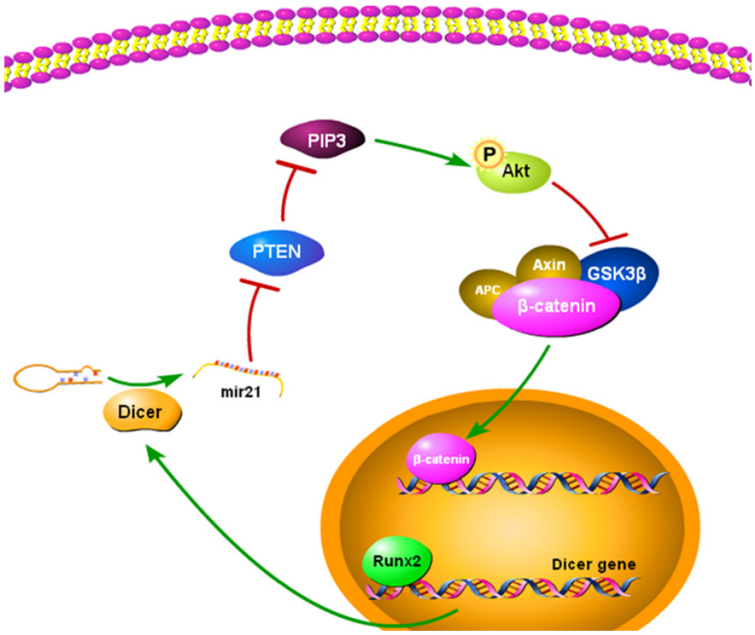

Figure 6. Summary of the mechanism of Dicer regulation in osteogenesis. Model demonstrates that Runx2 directly regulates Dicer expression to improve osteogenesis by up-regulating miR21a-5p targeting PTEN through activating Akt/Gsk3β/β-catenin signaling pathway.

Dicer knockdown cells significantly (albeit incompletely) rescued osteoblast mineralization (**Figure 5A**). Similar trends were also observed on the mRNA and protein levels in MC3T3-E1 cells treated the same as above determined by real time-PCR and western-blot (**Figure 5B**, **5C**), suggesting miR-21a-5p targeting PTEN may play a role in lineage commitment, although it is likely that other miRNAs as well as osteogenic regulators also contribute to control of osteogenesis. To explored the role of PTEN in the Akt/GSK3 signaling pathway of Dicer knockdown cells during osteogenesis, MC3T3-E1 cells were treated with siDicer or VO-OHpic trihydrate and followed by osteogenic induction. Western blot analysis revealed that Dicer knockdown increased the expression of PTEN and inhibited the phosphorylation of Akt and GSK-3β on ser9. Abatement was also observed in β-catenin in Dicer knockdown cells compared with control cells during the differentiation. In turn, we also verified that inhibitor of PTEN VO-OHpic trihydrate can promote the phosphorylation of Akt and GSK-3β on ser9 and then increased the accumulation of β-catenin in Dicer knockdown cells (**Figure 5D**). These results demonstrated that miR-21a-5p and PTEN may participate in osteogenesis by modulating Akt/GSK3β signaling to regulate the stabilization of β-catenin, which would act as a transcriptional co-factor to activate osteogenesis.

Discussion

Bone formation is a complex biological process requiring the interaction of transcription factor, signaling pathway and post-transcriptional system. However, the post-transcriptional regulatory mechanism underlying osteogenesis is limited. The microRNAs have been demonstrated to mediate protein translation in bone homeostasis. In the present study, we found Dicer expression increased during the osteogenesis, which in parallel to the tendency of Runx2 expression. Meanwhile, Dicer expression regulated by Runx2 in a dose-dependent manner was further validated by overexpression or knockdown of Runx2 in MC3T3-E1 cells. Furthermore, Knockdown of Dicer could attenuate the osteogenesis of pre-osteoblast MC3T3-E1 cells and the miR21a/PTEN/Akt/GSK3β/β-catenin regulation might be the mechanism involved (**Figure 6**).

Presently, numerous of miRNAs that play a role in bone development have been reported. They regulate the progression and timing of osteoblast maturation programs by expressing during stages of differentiation and inhibiting their target genes including both suppression and activation of osteogenesis. Studies show a group of miRNAs functionally promotes osteogenesis including miR-335-5p targeting DDK1 [28], miR-20a/b targeting PPARc [29, 30], miR-15 targeting Smurf1 [31], miR-214 down-regulated PTEN in RAW 264.7 [32] and miR-let-7 down-regulate Axin2 in hMSCs [33]. Additionally, a class of miRNAs have been identified as osteogenesis inhibitor by controlling their cognate target gene respectively, such as miRNA-34c, miRNA-133a, miRNA-135a, miRNA-137 suppressing Runx2 in

MC3T3-E1 cell lines [34], miRNA-23a-27a-24-2 targeting SATB2 [35], miRNA-31, miRNA-93 targeting Osx in hMSCs or MC3T3-E1 [36, 37] and miRNA-29a/b inhibiting COL1A1 and Col5A3 [38, 39]. However, the global function of miRNAs on bone metabolism confused us. RNase III endonuclease Dicer is a key processor of miRNAs biogenesis. Therefore, research of Dicer is an approach for understanding tissue specific requirements for miRNAs in osteogenesis. In agreement with previous studies [20, 35], our data presented the expression of osteogenic marker genes, such as Runx2, ALP, osterix, osteocalcin and osteopontin, were positively regulated by osteogenic induction. Although the specific time points chosen in our study and others were not exactly the same, the temporal changes in the expression of osteogenic marker genes observed were similar. The observed differences might have been caused by the differences in the cell lines that were used, as well as the culture conditions for osteogenesis. Nonetheless, we successfully constructed a model of osteogenesis for preosteoblasts. The expression trend of marker genes could support experimental evidence for selecting time points to succeeded research. Increase in the mRNA and protein levels of Dicer was parallel to the increase of Runx2 during osteogenic differentiation, which suggested that Dicer plays a positive role in the differentiation and has significant correlation with Runx2.

Osteoblast commitment and differentiation are influenced on the suitable expression of Runx2 regulating a series of osteogenic marker genes by binding to their cis-regulatory DNA elements [40]. Our previous study revealed DICER expression decreased in embryos of Runx2 knockout mice (Runx2 -/-) compared with Runx2 +/- and Runx2 +/+ mice. Runx2 significantly increased the activity of luciferase reporter in Dicer promoter was also showed [21]. In this study, dual luciferase reporter assays revealed Runx2 increasing Dicer gene promoter activity in a dose dependent manner and Runx2 directly binding to the promoter region of Dicer gene in preosteoblast at early stage of osteogenesis was confirmed by Chromatin immunoprecipitation assay, which supporting the hypothesis. Steven Pregizer performed a series of ChIP assays during osteogenesis revealed that Runx2 is recruited to the OCN promoter with a remarkably similar temporal pattern [18]. Our study took the same primers specific for the mouse OCN gene promoter region as positive control to amplify the purified DNA segments of our samples and found Runx2 occupancy of OCN promoter increased than control. Despite their results found the remarkably occupancy increased between days 4 and 11, our results was consistent with mRNA and protein levels of Dicer and Runx2 as discussed in the previous section.

Although Dicer plays a key role in a wild scope of biological processes, including cell growth, lineage commitment and cell apoptosis, through processing of microRNAs maturation [41], knowledge of the function and regulatory mechanisms underlying the role of Dicer in skeletal development and bone remolding are still emerging. Tripti Gaura performed knockout of Dicer in mice at two periods of bone formation and found col1-Cre depletion of Dicer in osteoprogenitors prevented their differentiation before birth and oc-Cre excision of Dicer delayed perinatal bone formation which control bone accrual in the adult. Their studies demonstrate that miRNAs are important for initiating osteoblast maturation during development and to regulate bone mass in adult mice [11]. Fumitaka Mizoguch conditionally excised Dicer in osteoclasts and found that the Dicer ablation-resulted osteoclastic suppression was dominant over Dicer ablation-resulted osteoblastic suppression, which accounted for the Dicer depletion in osteoclasts increased the amount of bone mass and indicated that Dicer controls the activity of bone resorption of osteoclasts in vivo [12]. Xuguang Nie et al identified neural crest cell-specific depletion of Dicer and their associated mesodermderived cells presented massive apoptosis, resulting severe abnormalities during craniofacial organogenesis and morphogenesis [14]. Tatsuya Kobayash conditionally disrupted the Dicer gene and found it led premature death and severe skeletal growth defects of mice, which indicated Dicer is essential for maintaining the chondrocytes proliferating pool through promoting chondrocyte proliferation and inhibiting premature chondrocyte differentiation to postmitotic hypertrophic chondrocytes. From the results obtained so far in our study, Dicer has been shown to exert a positive effect on the bone formation [13]. Our previous study transfected siDicer into bone marrow stromal cells (BMSCs) and transplanted it into

calvarial bone critical-size defect mouse, which revealed weaker bone formation in group of calvarial defects with the siDicer by micro-CT analysis. These facts suggest us to conduct an intensive study on the role of Dicer specifically in osteogenesis. Of note, as Dicer is required for the generation of its target small interfering RNA, these lentivirus or shRNA vectors were equivocal to be employed to eliminate Dicer expression. Therefore, siDicer was used to knockdown its expression in MC3T3-E1 cells. The impaired mineralization ability and decrease of osteogenic markers in Dicer knockdown group further confirm the promotive effect of Dicer in osteogenesis. These findings suggest that a cohort of miRNAs processed by Dicer participate in osteoblast phenotype differentiation and bone development.

Since numerous genes are predicted to be negatively regulated by miRNAs, it is expected that Dicer knockdown would affect multiple genes. MiR-21a-5p and Dicer simultaneous up-regulation along with PTEN protein down-regulation in osteogenesis in this study, suggesting that miR-21a-5p may regulate osteogenesis through depleting PTEN. TINGTING WU identified that miR-21, targeting PTEN, inhibits TGF-β1 expression in BMMSCs through activation of NF-κB pathway [27]. ADELE RICHART, also revealed miR-21a could modulate the pro-angiogenic potential of progenitor cells and the distinct steps through targeting PTEN/HIF-1a/VEGF-A signaling [26]. In our work, restoration miR-21a only partially diminished the suppression of osteogenesis by Dicer knockdown, which suggests that additional pathways are very likely to be involved in differentiation. PTEN, functions as a protein tyrosine phosphatase, dephosphorylates phosphatidylinositol-3, 4, 5-trisphosphate to phosphatidylinositol 4, 5-bisphosphate, which negatively regulate PI-3K/Akt signalings [42]. GSK3β is a key kinase that is at the intersection,of the PI3K/Akt and Wnt/β-catenin signaling pathways. Wnt signaling often tightly regulated for bone formation [44]. On the canonical Wnt pathway, the nuclear β-catenin acts as transcriptional co-activator for the enhancer factor and activates transcription of genes necessary for differentiation [45]. In contrast with the decrease of PTEN expression, β-catenin increased during the osteogenesis in our study. This implied that Dicer knockdown could affect PTEN by miR21a-5p and then regulate the phosphorylation of GSK-3β through PI3K/Akt signaling, resulting in the alteration of Wnt/β-catenin signaling and influence osteogenesis. Considering the alteration of pAKT, pGSK3β on ser9 and β-catenin detecting by western-blot, we inferred that pAKT, pGSK3β functions through PI3 kinase/AKT Signaling pathway. GSK-3β is inactivated via phosphorylation on Ser-9 and cause in the activation of Wnt/β-catenin signaling. Once β-catenin levels accumulate enough to translocate into the nucleus, it will engage upon the transcription factors to mediate gene expression. Moreover, the reduction of pAKT, pGSK3β and β-catenin caused in Dicer knockdown cells during osteogenesis could be rescued by PTEN inhibitor VO-OHPIC. Taken together, these findings indicated that Dicer could regulate osteogenesis through mir21/PTEN/pAKT/pGSK3β/β-catenin signaling pathway.

In conclusion, Dicer expression was found to increase in the cytoplasm, which was coincident with the expression of Runx2 protein in nucleus during osteogenesis. Exogenous expression of Runx2 transiently enhanced Dicer expression and knockdown of Runx2 by siRNA down-regulated Dicer expression. Runx2 can directly bind to the Dicer promoter site and activate promoter activity of Dicer to specifically regulate Dicer expression. Furthermore, these findings suggest that Dicer plays a critical role in improving osteoblast differentiation and the pathway consisting of mir21a/PTEN/pAKT/pGSK3β/β-catenin participates in a lineage commitment of the role of Runx2 and Dicer in cell osteogenesis. These results will help us to understand the role of Dicer in osteogenesis and may provide a new potential therapeutic target for bone tissue regeneration.

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Disclosure of conflict of interest

None.

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