MicroRNA expression analysis during FK506-induced osteogenic differentiation in rat bone marrow stromal cells

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Abstract. FK506 (also known as tacrolimus) is a potent immunosuppressive agent that is widely used in the treatment of graft-rejection and autoimmune diseases. FK506 has attracted additional attention owing to its potential role in osteogenic differentiation and bone formation. MicroRNAs (miRNAs) have been demonstrated to serve important roles in the regulation of osteogenic differentiation; however, identification of specific miRNAs and their roles in regulating FK506-induced osteogenic differentiation have been poorly examined. In the present study, osteodifferentiation of rat bone marrow stromal cells (BMSCs) was induced with varying concentrations of FK506 (5-5,000 nM) for 3, 7 and 14 days. Differentially expressed miRNAs were profiled using miRNA array, verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and subjected to gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Results from the present study identified a subset of miRNAs that were differentially expressed, of which five upregulated miRNAs (miR-106b-5p, miR-101b-3p, miR-193a-3p, miR-485-3p and miR-142-3p) and four downregulated miRNAs (miR-27a-3p, miR-207, miR-218a-2-3p and let-7a-5p) were confirmed by RT-qPCR. GO and KEGG analysis revealed that the predicted target genes of these miRNAs are involved in multiple biological processes and signaling pathways, including cell differentiation and the mitogen-activated protein kinase (MAPK) signaling pathway. Verification of the miRNA-target genes revealed that Smad5, Jagged 1 and MAPK9 were significantly upregulated, whereas Smad7, BMP and activin membrane-bound inhibitor, and dual-specificity phosphatase 2 were significantly downregulated during FK506-induced osteodifferentiation. The present study may provide an experimental basis for further research on miRNA functions during FK506-induced osteogenic differentiation in rat BMSCs.

Introduction

FK506 (also known as tacrolimus) is a potent immunosuppressive agent that is used to prevent graft rejection and to treat autoimmune diseases, both experimentally and clinically (1). Over the past decade, several studies have reported that FK506 treatment may increase bone mass and may be a potential treatment for bone defects (2,3). FK506 exposure has been reported to induce osteogenic differentiation by promoting the expression of osteoblastic transcription factors, including core-binding factor α1 (also termed runt related transcription factor 2, Runx2), osteopontin (OPN) and osteocalcin (OCN) (2,3). A previous in vivo study using bone tissue/hydroxyapatite composite demonstrated increased bone formation and higher osteogenic parameters when cultured with FK506 (4). However, other studies reported that FK506 may result in osteoporosis by increasing the expression levels of osteoclast differentiation factor or by inhibiting osteoblast differentiation by suppressing the activity of calcineurin and the expression of Runx2 (5,6). These results suggested that the function of FK506 during osteogenic differentiation was contradictory and required for further study.

MicroRNAs (miRNAs) are small, 22-nucleotides-long, endogenous noncoding RNAs that bind to target mRNAs at the 3′-untranslated region to mediate translation and reduce protein expression levels (7). They are key negative regulators of diverse biological and pathological processes, such as proliferation, migration, apoptosis and differentiation (7,8). A number of miRNAs have been demonstrated to be involved in the differentiation of osteoblasts from bone marrow stromal cells (BMSCs) by regulating specific gene expression (9). One previous study has indicated that miRNA (miR)-96 may promote osteogenic differentiation by suppressing the expression of heparin-binding epidermal growth factor-like growth factor in mouse BMSCs (10). During the process of osteogenic differentiation of BMSCs, miR-205 was downregulated and inhibition of miR-205 enhanced osteogenic differentiation by regulating the expression of special AT-rich sequence-binding 2 (SATB2) and Runx2 (11). However, identification of specific miRNAs and their regulatory roles in the FK506-induced osteodifferentiation have been poorly investigated.

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The aim of the present study was to examine the effects of various concentrations of FK506 (5-5,000 nM) on the osteogenic differentiation of rat BMSCs. Differentially expressed miRNAs were profiled by miRNA array, verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and subjected to gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Materials and methods

Isolation and culture of rat BMSCs. Primary BMSCs were harvested from 5 male Sprague Dawley rats (age, 4-5 weeks; weight, 80-100 g) using previously described methods (2). The male Sprague-Dawley rats were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were maintained in a temperature-controlled room (22±3℃) with a 12-h light/dark cycle and were allowed free access to drinking water and food. All procedures were approved by The Animal Research Committee of Zhongshan Hospital, Fudan University (Shanghai, China). Briefly, rats were sacrificed by cervical dislocation followed by soaking in 75% ethanol for 10 min. The tibia and femur were aseptically dissected and the two ends of each bone were cut using aseptic conditions. The marrow was flushed out with rat Mesenchymal Stem Cell Growth medium (Cyagen Biosciences, Inc., Santa Clara, CA, USA) containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glucose. The cells were centrifuged at 500 x g for 5 min at 4˚C. Cells were diluted with cell number was determined by cell counting assay following manufacturer's instructions (Precision Instrument Co. Ltd., Tokyo, Japan) inverted microscope equipped with a digital camera and connected to a PC using MagnaFire 2.0 camera software (Optronics, Goleta, CA, USA).

Phenotype analysis. BMSC cell-surface markers were analyzed by flow cytometry. Briefly, cells at passage 3 with 80% confluence were trypsinized using TrypLE Express (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and subcultured to passage 3 for further analysis. The cells were analyzed using an Olympus SZX12 (Olympus Corporation, Tokyo, Japan) inverted microscope equipped with a digital camera and connected to a PC using MagnaFire 2.0 camera software (Optronics, Goleta, CA, USA).

RNA extraction and RT-qPCR analysis. According to the manufacturer’s protocol, total RNA was extracted from 1x10^6 control and FK506-induced BMSCs with TRIzol Reagent (Invitrogen, Carlsbad, USA), purified using the RNeasy Mini Kit (Qiagen, CA, USA) and quantitated by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA (500 ng) was reverse transcribed into cDNA using the PrimeScript™ RT-PCR kit (Takara Bio, Inc., Otsu, Japan). The cDNA was amplified using the SYBR Premix Ex Taq II kit (Takara Bio, Inc.) with the GeneAmp-PCR system 7500 (Thermo Fisher Scientific, Inc.). Total RNA for miRNA analysis was isolated using a miRCute miRNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China) and the expression of mature miRNA was determined by miRCute miRNA PCR Detection kit (Tiangen Biotech Co., Ltd.) with the GeneAmp-PCR system 7500. All primers used for RT-qPCR are listed in the Table I, and each sample was measured in triplicate. Gene expression results of mRNA or miRNA were evaluated by the 2^-ΔΔCq method (13) and normalized to GAPDH or U6, respectively.
miRNA microarray analysis. miRNA microarray analysis was performed by KangChen Bio-tech Inc. (Shanghai, China). Briefly, total RNA was extracted from 1x10^7 control and FK506-induced BMSCs using TRIzol reagent and purified with RNAeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to manufacturer's protocol. The quality and quantity of RNA were measured by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was determined using 1.5% gel electrophoresis. miRNAs were labeled with Hy5 fluorescence dyes using the miRCURY LNA microarray kit (Exiqon A/S, Vedbaek, Denmark) and hybridized in a Hybridization Chamber II (Ambion; Thermo Fisher Scientific, Inc.) using the miRCURY LNA microarray kit (Exiqon A/S) according to the manufacturer's protocol. Following hybridization, the microarray slides were processed with an Axon GenePix 4000B Microarray Scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA), and data analysis was performed using GenePix Pro software version 6.0 (Molecular Devices, LLC). The experiment was performed with 3 samples both in control and treatment group, and miRNAs were defined as differentially expressed if changes in expression levels were ≥2-fold than those of the controls.

miRNA microarray analysis.

GO analysis and KEGG pathway annotation based on miRNA expression profile. Target genes of the differentially expressed miRNAs were predicted using miRBase (http://www.mirbase.org), miRanda (http://www.microrna.org), TargetScan (http://www.targetscan.org) and miRDB (http://mirdb.org/miRDB) databases. GO analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov) to determine the functions of the predicted target genes and to uncover the miRNA-target gene regulatory network based on the predicted biological processes and molecular functions. KEGG pathway analysis (http://www.genome.jp/kegg) was used to determine the potential pathways that the predicted target genes may be a part of. Fisher's exact test and the \( \chi^2 \) test were used to classify the GO category and KEGG pathway, and the false discovery rate (FDR) (14) was calculated to correct the P-value. Thresholds of P<0.05 and FDR<0.05 were used to select significant GO categories. Fisher P-value stands for the enrichment P-value used Fisher's exact test and Fisher-P value<0.05 was used as a threshold to select significant KEGG pathways.

Statistical analysis. Statistical analysis of two groups was determined by unpaired Student's t-test. All data were represented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Growth characteristics and phenotype of BMSCs using flow cytometry. Following 3 days of primary culture, the isolated BMSCs were mostly spindle shaped, single or small colony size and grew at a low density. (Fig. 1A). Primary BMSCs cultured for 12 days replicated rapidly and reached ~80% confluence (Fig. 1B). Cells were expanded by successive subculture and exhibited colony-like growth at passage 3. BMSCs phenotypes were verified by flow cytometric analysis. The subcultured BMSCs at passage 3 were positive for MSCs markers CD29 (99.9%) and CD90 (99.3%), and negative for hematopoietic lineage markers CD34 (0.998%) and CD45 (0.064%) (Fig. 1C).

FK506-induced osteogenic differentiation of BMSCs. To detect the effects of FK506 treatment on the osteogenic differentiation of BMSCs, cells were collected at passage 3 and cultured in osteo-induction medium with a concentration of FK506 ranging between 5 and 5,000 nM. ALP and ARS staining were strongest in cells treated with 50 nM FK506 of FK506 ranging between 5 and 5,000 nM. ALP and ARS staining were strongest in cells treated with 50 nM FK506 (Fig. 2A). RT-qPCR analysis of BMSCs treated with 50 nM FK506 revealed that the expression of Sp7, Runx2, and OPN were significantly increased following 7 days stimulation and the \( \chi^2 \) test was used to determine the potential pathways that the predicted target genes may be a part of. Fisher's exact test and Fisher-P value<0.05 was used as a threshold to select significant KEGG pathways.

miRNA expression analysis following FK506-induced osteogenic differentiation. To examine the differential expression of miRNAs that are involved in FK506-induced osteogenic differentiation.
Figure 1. Growth characteristics and phenotypic analysis of rat BMSCs. Inverted microscope images (40x) reveal that BMSCs in primary culture were (A) mostly spindle-shaped and single cells at day 3 and (B) reached almost 80% confluence at day 12. Flow cytometric analysis indicated that BMSCs at passage 3 were positive for CD29 and CD90, and negative for CD34 and CD45 (C). BMSCs, bone marrow stromal cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Figure 2. ALP and ARS staining in BMSCs after FK506-induced osteogenic differentiation. (A) ALP and ARS staining were strongest in rat BMSCs when stimulated with FK506 at the concentration of 50 nM. (B) Expression levels of osteoblastic specific genes were significantly increased at 50 nM FK506 treatment for 7 days. *P<0.05 and **P<0.01 compared with NC. Data are expressed as the mean ± standard error of the mean; n=3 per group. ALP, alkaline phosphatase; ARS, alizarin red S; d, days; NC, negative control; OCN, osteocalcin; OPN, osteopontin; Runx2, runt-related 2.
osteodifferentiation of rat BMSCs, miRCURY LNA microarrays were used to profile miRNA expression 7 days following FK506 (50 nM) induction. Comparison with the control group identified a total of 56 miRNAs, including 17 that were upregulated and 39 that were downregulated (Tables II and III, respectively). RT-qPCR analysis was used to verify the microarray results and demonstrated that five miRNAs were upregulated (miR-106b-5p, miR-101b-3p, miR-193a-3p, miR-485-3p and miR-142-3p) and four were downregulated (miR-27a-3p, miR-207, miR-218a-2-3p and let-7a-5p) compared with untreated control cells (Fig. 3).

GO category and KEGG pathway analysis. To investigate the specific roles of the nine identified differentially expressed miRNAs in the regulation of osteodifferentiation in BMSCs, target genes were predicted and subjected to GO term and KEGG pathway analysis. GO analysis demonstrated that the highly enriched GO terms targeted by these dysregulated miRNAs were involved in multiple biological processes such as cell differentiation, cell growth and cell migration (Tables IV and V). KEGG pathway analysis revealed the target genes to be highly enriched in 22 upregulated and 21 downregulated signaling pathways, including mitogen-activated protein kinase (MAPK) signaling pathway, calcium signaling pathway and Toll-like receptor signaling pathway (Fig. 4).

Verification of the target genes of differentially expressed miRNAs. Osteodifferentiation is driven by a series of complex events that result in the activation of specific transcription factors. Of the predicted target genes and differentially regulated function analysis, only those closely related to osteoblast differentiation, ossification and signal transduction are listed in Table VI. RT-qPCR demonstrated that MAPK9, Smad5 and jagged 1 (Jag1), which are positive regulators of osteogenesis, were significantly upregulated in FK506-induced rat BMSCs undergoing osteogenic differentiation, compared with the untreated control (Fig. 5); whereas dual-specificity phosphatase 2 (Dusp2), Smad7 and BMP and activin membrane-bound inhibitor (Bambi), which are negative regulators of osteodifferentiation, were demonstrated to be significantly downregulated.

| microRNA          | Fold change |
|-------------------|-------------|
| mo-miR-27a-3p     | 2.70        |
| mo-miR-218a-2-3p  | 3.70        |
| mo-miR-665        | 5.00        |
| mo-miR-542-5p     | 10.0        |
| mo-miR-207        | 2.63        |
| mo-miR-329-3p     | 3.45        |
| mo-miR-10a-5p     | 3.03        |
| mo-miR-450a-5p    | 4.12        |
| no-miR-672-3p     | 3.45        |
| no-miR-878        | 3.03        |
| no-miR-2985       | 2.56        |
| no-miR-370-5p     | 2.38        |
| no-miR-488-3p     | 3.23        |
| no-miR-193a-5p    | 6.67        |
| no-miR-29b-3p     | 2.17        |
| no-miR-465-5p     | 2.33        |
| no-miR-3560       | 2.13        |
| no-miR-3596d      | 3.70        |
| no-miR-1956-5p    | 50.0        |
| mo-miR-653-3p     | 4.76        |
| mo-miR-203a-3p    | 7.14        |
| mo-miR-652-5p     | 6.25        |
| mo-miR-200c-5p    | 4.17        |
| mo-miR-181b-5p    | 2.13        |
| mo-miR-1-3p       | 2.04        |
| mo-miR-143-5p     | 2.70        |
| mo-miR-664-2-5p   | 2.38        |
| mo-let-7a-5p      | 2.22        |
| mo-miR-3557-5p    | 2.08        |
| mo-miR-331-5p     | 3.85        |
| mo-let-7f-5p      | 3.57        |
| mo-miR-18a-3p     | 12.5        |
| mo-miR-30c-1-3p   | 2.33        |
| mo-miR-10a-3p     | 25.0        |
| mo-miR-487b-3p    | 2.38        |
| mo-miR-497-5p     | 11.1        |
| mo-miR-582-5p     | 2.56        |
| mo-miR-493-5p     | 3.45        |
| mo-miR-194-3p     | 5.00        |

miR, microRNA; mo, Rattus norvegicus.
The results suggested that FK506 treatment may induce osteogenic differentiation by regulating the expression of specific target genes of these differentially expressed miRNAs.

Discussion

FK506 is a powerful and clinically useful immunosuppressant, which has been widely used as a treatment in organ transplantation, atopic dermatitis and rheumatoid arthritis (15). During the treatment of rheumatoid arthritis, a number of studies demonstrated that FK506 treatment could increase bone formation, inhibit osteoclastic bone resorption and improve clinical symptoms (16,17). In addition, it has been reported that FK506 exposure may promote osteogenic and chondrogenic differentiation (18,19), and a change of immunosuppressive monotherapy from cyclosporin A to FK506 may be able to reverse bone loss (20), indicating that FK506 may be a potential treatment for bone defects.
Table V. Predicted GO term enrichment of the downregulated microRNAs in FK506-induce osteodifferentiation in rat bone marrow stromal cells.

| GO.ID          | Category name                                      | Fold enrichment | P-value     | FDR         |
|----------------|----------------------------------------------------|-----------------|-------------|-------------|
| GO:0044710     | Single-organism metabolic process                  | 1.55            | 4.56x10^-10 | 8.19x10^-7 |
| GO:007102      | Organic substance transport                         | 1.98            | 5.32x10^-9  | 5.73x10^-6 |
| GO:0010648     | Negative regulation of cell communication          | 2.32            | 4.75x10^-7  | 1.47x10^-4 |
| GO:0042981     | Regulation of apoptotic process                     | 2.00            | 3.06x10^-6  | 5.15x10^-4 |
| GO:0044237     | Cellular metabolic process                          | 1.21            | 2.25x10^-5  | 2.02x10^-3 |
| GO:0048583     | Regulation of response to stimulus                 | 1.52            | 3.12x10^-5  | 2.58x10^-3 |
| GO:0006464     | Cellular protein modification process               | 1.53            | 3.37x10^-5  | 2.63x10^-3 |
| GO:0030182     | Neuron differentiation                              | 1.92            | 3.82x10^-5  | 2.94x10^-3 |
| GO:0050767     | Regulation of neurogenesis                          | 2.31            | 5.02x10^-5  | 3.57x10^-3 |
| GO:0007399     | Nervous system development                          | 1.65            | 5.04x10^-5  | 3.57x10^-3 |
| GO:0050804     | Regulation of synaptic transmission                | 3.04            | 5.12x10^-5  | 3.58x10^-3 |
| GO:0065009     | Regulation of molecular function                    | 1.59            | 5.81x10^-5  | 3.89x10^-3 |
| GO:0016049     | Cell growth                                         | 2.62            | 9.00x10^-5  | 5.25x10^-3 |
| GO:2000026     | Regulation of multicular organism development       | 1.73            | 9.04x10^-5  | 5.25x10^-3 |
| GO:0044249     | Cellular biosynthetic process                       | 1.34            | 9.74x10^-5  | 5.35x10^-3 |
| GO:0030799     | Regulation of cyclic nucleotide metabolic process   | 4.18            | 1.44x10^-4  | 7.08x10^-3 |
| GO:0006954     | Inflammatory response                               | 2.38            | 1.69x10^-4  | 7.71x10^-3 |
| GO:0031100     | Organ regeneration                                  | 4.52            | 1.72x10^-4  | 7.71x10^-3 |
| GO:0043269     | Regulation of ion transport                         | 2.25            | 1.97x10^-4  | 8.51x10^-3 |
| GO:0042127     | Regulation of cell proliferation                    | 1.72            | 2.86x10^-4  | 9.77x10^-3 |
| GO:0045595     | Regulation of cell differentiation                  | 1.68            | 4.15x10^-4  | 1.31x10^-2 |
| GO:0023014     | Signal transduction by phosphorylation              | 2.07            | 4.87x10^-4  | 1.47x10^-2 |
| GO:0055012     | Ventricular cardiac muscle cell differentiation      | 10.00           | 5.82x10^-4  | 1.67x10^-2 |
| GO:0051592     | Response to calcium ion                             | 3.77            | 6.68x10^-4  | 1.84x10^-2 |
| GO:0000165     | MAPK cascade                                        | 1.99            | 1.43x10^-3  | 3.16x10^-2 |
| GO:0017156     | Calcium ion-dependent exocytosis                   | 4.78            | 1.56x10^-3  | 3.31x10^-2 |
| GO:0030099     | Myeloid cell differentiation                        | 2.33            | 2.16x10^-3  | 4.00x10^-2 |
| GO:0035051     | Cardiocyte differentiation                          | 3.35            | 2.78x10^-3  | 4.78x10^-2 |
| GO:0002520     | Immune system development                           | 1.76            | 2.81x10^-3  | 4.78x10^-2 |
| GO:0045622     | Regulation of T-helper cell differentiation          | 10.00           | 2.98x10^-3  | 4.85x10^-2 |

FDR, false discovery rate; GO, gene ontology; MAPK, mitogen-activated protein kinase.

Figure 4. KEGG pathway analysis for predicted target genes. (A) Pathways that may be upregulated in FK506-induced rat BMSCs. (B) Pathways that may be downregulated in FK506-induced rat BMSCs. The horizontal axis is the enrichment of pathways and the vertical axis is the pathway category. Fisher-P-value<0.05 was used as a threshold to select significant KEGG pathways. LgP is the negative logarithm of the Fisher-P-value. ABC, ATP-binding cassette; BMSCs, bone marrow stromal cells; GABA, γ-aminobutyric acid; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor.
Table VI. Predicted target genes and functions of microRNAs associated with FK506-induced osteodifferentiation in rat bone marrow stromal cells.

| microRNA       | Target genes          | Function                                      |
|----------------|-----------------------|-----------------------------------------------|
| rno-miR-101b-3p| Smad5                 | Osteoblast differentiation, ossification      |
|                | TGFβR1                | Receptor for growth factor, protein metabolism |
| rno-miR-106b-5p| Bambi                 | Stem cell differentiation, TGF-β receptor signaling |
|                | Dusp2                 | Tissue development, signal transduction       |
| MAP3K2, MAP3K8 and MAPK9 | Smad7 | MAPK cascade, proliferation and differentiation |
| rno-miR-142-3p| TGFβR1                | Receptor for growth factor, protein metabolism |
| rno-miR-27a-3p| MAPK9                 | MAPK cascade, proliferation and differentiation |
|                | Smad5                 | Osteoblast differentiation, ossification      |
|                | CBFβ                  | Osteoblast differentiation, osteogenesis      |
| rno-miR-218a-2-3p| Wnt5a | Stem cell differentiation and proliferation |
|                | Jag1                  | Mesenchymal cell differentiation, osteogenesis |
| rno-let-7a-5p | TGFβR1 | Receptor for growth factor, protein metabolism |
|                | AcvR1C               | MAPK cascade, proliferation and differentiation |
| MAP6 and MAPK9 |                       |                                               |

AcvR1c, activin A receptor type 1C; Bambi, BMP and activin membrane-bound inhibitor; BMP, bone morphogenetic protein; CBFβ, core-binding factor β; Dusp2, dual-specificity phosphatase 2; Jag1, jaggled 1; MAPK, mitogen-activated protein kinase; MAP3K, mitogen-activated protein kinase kinase kinase; miR, microRNA; rno, Rattus norvegicus; TGF, transforming growth factor; TGFβR1, TGFβ receptor 1.

However, other studies demonstrated that FK506 treatment may result in osteoporosis, prevent osteoblast differentiation and/or cause significant bone loss (5,6,21). These conflicting results may be due to different drug concentrations administered or induction time, which is crucial in the process of bone metabolism. Low-dose and short-term application of FK506 may be able to promote the early stage of osteogenesis. By contrast, long-term administration of FK506 led to accelerated bone formation and bone resorption (22). The present study demonstrated that FK506 treatment in rat BMSCs had the most significant osteogenic potential when administered at a dose of 50 nM for 7 days; whereas high concentrations of FK506 or longer periods of stimulation reduced the osteogenic potential, suggesting that the possible side effects of drug cytotoxicity may inhibit the osteoinduction of BMSCs.

miRNAs serve a key role in regulating osteodifferentiation (9). The present study identified and verified five upregulated and four downregulated miRNAs that were differentially expressed in FK506-induced rat BMSCs undergoing osteogenic differentiation, some of which have been reported as positive and/or negative regulators of osteogenesis. For example, miR-27a, an early negative regulator of osteogenesis, was revealed to delay osteoblast differentiation by suppressing SATB2 expression (23). By contrast, a different study reported that miR-27a may be able to promote osteoblast differentiation by repressing the expression of secreted frizzled-related protein at the transcriptional level (24). A previous study revealed that miR-193a was downregulated during osteogenic differentiation of human adipose-derived stem cells and the osteoporotic fractures; however, the mechanism has not been elucidated (25). In addition, miR-218 was reported to be upregulated during osteodifferentiation and its overexpression could significantly increase human adipose tissue-derived stem cells osteogenic differentiation (26). However, results from the present study are not fully in line with the results of previous studies, which may be explained by the variability of mesenchymal stem cells from different tissues and culture microenvironments.

To further investigate the molecular mechanisms of FK506-induced osteogenic differentiation in rat BMSCs, functional analysis were performed. Predicted miRNA target genes, such as MAPK9 and Dusp2, were verified by RT-qPCR. Highly enriched KEGG pathway analysis indicated that MAPK9 and Dusp2 may serve a role through the MAPK signaling pathway, which is involved in a number of cellular functions, such as differentiation, proliferation and cell death (27). Of these, the physiological functions of MAPK9 [also known as c-Jun N-terminal kinase 2 (JNK2)] in osteodifferentiation and bone formation have been investigated previously (28). MAPK9 activity is essential for the expression of activating transcription factor 4, and its overexpression may enhance mineral deposition that is essential in the late stages osteogenic differentiation (29). Activation of JNKs is also essential for bone morphogenetic protein (BMP) 9-induced osteodifferentiation of mesenchymal stem cells (30). The expression of Dusp2, an inhibitor of osteoblast differentiation, was revealed to be downregulated during osteogenic differentiation (31,32). The DUSP subfamily (DUSP1, DUSP2, DUSP4 and DUSP5) of nucleus-inducible MAPK phosphatases are rapidly induced by growth factors or stress signals, such as mitogens, oxidative stress, heat shock and hypoxia and localize exclusively to the nucleus (32,33). In the present study, MAPK9 expression was revealed to be significantly upregulated in rat BMSCs stimulated with...
were identified as differentially expressed and may regulate osteogenic differentiation of rat BMSCs at the concentration demonstrated that FK506 treatment was able to significantly promote proliferation, differentiation and mineralization of mouse myoblast cells was demonstrated to cause the inability to undergo BMP-induced osteoblast differentiation and Smad1/5-knockout mice exhibited severe chondrodysplasia. Smad7 and Bambi act as negative regulators of osteogenic differentiation: Smad7 was reported to inhibit proliferation, differentiation and mineralization of mouse osteoblastic cells and Bambi, a target gene of miR-20a, was demonstrated to inhibit the osteogenesis of human BMSCs. In the present study, although the target genes of differentially expressed miRNAs were not highly enriched in the TGF-β/Smad signaling pathway, it may be concluded that these differentially expressed miRNAs may promote FK506-induced osteogenic differentiation by regulating the expression of their target genes, such as Smad5, Smad7 and Bambi.

In conclusion, the results of the present study demonstrated that FK506 treatment was able to significantly promote osteogenic differentiation of rat BMSCs at the concentration of 50 nM. Following 7 day stimulation, several miRNAs were identified as differentially expressed and may regulate osteodifferentiation by targeting genes that are involved in bone formation. These preliminary results may provide an experimental basis for further research on the functions of miRNAs in FK506-induced osteogenic differentiation.

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