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MicroRNA expression profiling of human bone marrow mesenchymal stem cells during osteogenic differentiation reveals Osterix regulation by miR-31

Serena Rubina Baglìo *, Valentina Devescovi, Donatella Granchi, Nicola Baldini

Laboratory for Orthopedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, via di Barbiano 1/10, 40136, Bologna Italy

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ABSTRACT

Osteogenesis is the result of a complex sequence of events that involve the differentiation of mesenchymal stem cells (MSC) into osteoblasts. MSCs are multipotent adult stem cells that can give rise to different cell types of the mesenchymal germ layer. The differentiation fate of MSCs depends on the microenvironmental signals received by these cells and is tightly regulated by multiple pathways that lead to the activation of specific transcription factors. Among the transcription factors involved in osteogenic differentiation Osterix (Sp7) plays a key role and has been shown to be fundamental for bone homeostasis. However, the molecular events governing the expression of this transcription factor are not fully understood.

In this study we set out to investigate the changes in the microRNA (miRNA) expression that occur during the osteogenic differentiation of bone marrow-derived MSCs. To this purpose, we analyzed the miRNA expression profile of MSCs deriving from 3 donors during the differentiation and mineralization processes by microarray. 29 miRNAs were significantly and consistently modulated during the osteogenic differentiation and 5 during the mineralization process. Interestingly, most of the differentially expressed miRNAs have been reported to be implicated in stemness maintenance, differentiation and/or oncogenesis. Subsequently, we focused our attention on the regulation of Osterix by miRNAs and demonstrated that one of the miRNAs differentially modulated during osteogenic differentiation, miR-31, controls Osterix expression through association to the 3′ untranslated region of this transcription factor. By analyzing miR-31 and Osterix expression levels we found an inverse miRNA-target expression trend during osteogenic differentiation and in osteosarcoma cell lines. Moreover, the inhibition of the microRNA activity led to an increase in the endogenous expression of Osterix.

Our results define a miRNA signature characterizing the osteogenic differentiation of MSCs and provide evidence for the involvement of miR-31 in the regulation of the bone-specific transcription factor Osterix.

1. Introduction

Over the past few years the importance of microRNAs (miRNAs) in almost every cellular process and related diseases has been established. These small non-coding RNAs exert their activity mainly by suppressing the expression of specific transcripts through binding the 3′UTR, thereby providing an additional level of gene regulation (Bartel, 2009; Huntzinger and Izaurralde, 2011).

Recently, an increasing number of research groups are focusing on addressing the involvement of miRNAs in osteogenic differentiation and bone development. Consequently, various miRNAs have been reported to influence the fate of bone precursors (Eskildsen et al., 2011; Li et al., 2008, 2009a,b; Oskowitz et al., 2008; Schoolmeesters et al., 2009). However, no comprehensive miRNA expression profiling of differentiating and mineralizing human MSCs is currently available.

Osteogenesis is the result of a sequence of well-coordinated and fine-tuned events, which drive the commitment and the differentiation of mesenchymal stem cells (MSC) into osteoblasts. MSCs are non-hemopoietic (CD34−), stroma-derived multipotent cells able to generate osteoblasts, adipocytes or chondrocytes *in vitro* (Dominici et

Abbreviations: 3′UTR, 3′ untranslated region; ALP, Alkaline phosphatase; AP-1, Activator protein 1; ATF4, Activating transcription factor 4; BGLAP, Bone gamma-carboxyglutamate (gla) protein; BMP, Bone morphogenetic protein; COL1A1, Collagen, type I, alpha 1; FBS, Fetal bovine serum; FGF, Fibroblast growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; hTR, Telomerase RNA component; KLF4, Kruppel-like factor 4; MD1, MSC differentiation 1; MD4, MSC differentiation 4; MiR-Vec, microRNA expressing vector; miRNA, microRNA; MM2, MSC mineralization 2; MSC, Mesenchymal stem cells; NFATc1, Nuclear factor of activated T-cells, cytoplasmic; Oct4, Octamer-binding transcription factor 4; Osterix-mut31-Sen-Luc, Osterix miR-31 mutant sensor luciferase vector; Osterix-Sen-Luc, Osterix sensor luciferase vector; RFU, Relative fluorescence units; Runx2, Runt related transcription factor 2; Sen-Luc, Sensor luciferase vector; Sox2, SRY (sex determining region Y)-box 2; Sox9, SRY (sex determining region Y)-box 9; Sp/XKLF, specificity protein/X Krüppel-like factor; Sp7, Sp7 transcription factor (Osterix); TCF/LEF, T-cell factor/lymphoid enhancing factor; TGF-β, Transforming growth factor, beta; Twist, Twist basic helix–loop–helix transcription factor 1; Wnt, Wingless signaling pathway.

* Corresponding author at: Deparment of Pathology, Cancer Center Amsterdam, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Tel.: +39 3402376866.

E-mail addresses: rubinabaglio@gmail.com (S.R. Baglìo), valentina.devescovi@gmail.com (V. Devescovi), donatella.granchi@ior.it (D. Granchi), nicola.baldini@ior.it (N. Baldini).

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al., 2006; Horwitz et al., 2005). In the MSC niche a variety of growth factors and signaling pathways act in concert to induce the acquisition of the different phenotypes by the common precursor, which resides predominantly in the bone marrow and fat, but also in tissues such as periosteum, skeletal muscle, synovium, dental pulp and umbilical cord (Rosenbaum et al., 2008).

Osteogenic differentiation is primarily driven by the activity of Wnt, TGF-β, BMP, FGF and Hedgehog signaling pathways. At the transcriptional level, different factors have been identified that play a key role in both the initial commitment and the subsequent differentiation of MSC, such as Runx2, Osterix, SMADs, TCF/LEF, NFATc1, Twist, AP-1 and ATF4. These factors do not act on their own, but interact and integrate diverse signals and fine-tune gene expression (Deng et al., 2008; Jensen et al., 2010; Karsenty, 2003). Runx2 is considered the first master transcription factor responsible for the acquisition of osteochondroblastic characteristics and for the concomitant repression of the potential of cells to differentiate towards the adipocytic phenotype (komori, 2006). Downstream of Runx2 the fate of the bone precursor is mainly determined by the expression of Osterix (Sp7) (Nakashima et al., 2002).

Osterix belongs to the Sp subfamily of Sp/XKLF transcription factors (Gao et al., 2004). It acts in a complex with NFATc1 (Koga et al., 2005) and activates the transcription of bone-related genes, among which collagen type 1 (COL1A1) and Osteocalcin (BGLAP). The importance of this bone-specific transcription factor in osteoblast differentiation and bone formation has been demonstrated in Osterix null mice, where, while the ability to form cartilage was retained, bone formation was impaired due to the complete absence of osteoblasts (Nakashima et al., 2002).

Molecular defects in the osteogenic differentiation, caused by genetic or epigenetic alterations, can disrupt normal bone formation and remodeling and have pathological consequences (Ho et al., 2000; Jensen et al., 2010). We previously performed a comprehensive analysis of protein-coding genes modulated in the course of osteogenic differentiation and mineralization as a tool to monitor the differentiative and functional state of MSC in pathological conditions and for tissue engineering applications (Granchi et al., 2010). With the miRNome analysis acquiring more and more importance in providing markers of physiological and pathological processes, we now intended to enrich our knowledge on the molecular players involved in osteogenesis. Therefore, the objective of this study was to investigate the miRNA expression profile of MSCs at crucial timepoints of osteogenic differentiation in which the majority of the changes in the expression of protein-coding genes were observed. Subsequently, given the relevance of Osterix activity in this process and its implication in bone diseases and cancer, the understanding of the molecular events regulating this transcription factor has been the center of our interest.

2. Materials and methods

2.1. Cell culture

MCF-7, SaOS2, MG-63 and U2OS cell lines were cultured in Dulbecco's modified Eagle's medium (MCF-7 cells; Invitrogen) or Iscove's medium (SaOS2, MG-63 and U2OS cells; GIBCO) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and maintained at 37 °C in a 5% CO_2-humidified atmosphere.

After obtaining the informed consent, the bone marrow of 4 donors was processed to obtain the mononuclear cells by means of the Ficoll Hystopaque density gradient method (Sigma-Aldrich). Cells were maintained in alpha modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.1 M ascorbic acid-2 phosphate (Sigma-Aldrich). After 4 days (time point t0) non-adherent cells were removed, and the adherent cells were re-fed with fresh medium with the addition of 10^{-8} M dexamethasone (Sigma-Aldrich) (differentiation medium). After the first confluence, 10 mM β-glycerophosphate (Sigma) was added to the culture medium (mineralization medium) of confluent MSC cultures to induce the formation of mineral nodules.

Table 1
Cloning and qRT-PCR primers.

Amplification product	Primer sequence (5′–3′)
Osterix 3′UTR	F: gcgcctcgaggttaagccgggtggaaggtctcccac R: gcgcgcggccgcgactccacaaagggcatgatccct
Osterix mut31 3′UTR (miRNA-31 mutant)	F: tcttggctctctagaccactgcatatgaatcactctctttaccccatgc R: gcatggggtaaagagagtgattcatatgcagtggtctagagagccaaga
Renilla luciferase-Osterix 3′ UTR	F: caggaggacgctccagatga R: gatggcatgcatggggtaaa
Firefly luciferase	F: ttcgctaagagcaccctgat R: gtaatcagaatggcgctggt

RNA was isolated at time points corresponding to the main events related to MSC differentiation, including time point 0 (MD1), full confluence of the culture after the addition of the differentiating medium (MD4) and mineral nodule deposition one week after the addition of the mineralization medium (MM2) (Granchi et al., 2010). MSC viability was assessed by Alamar Blue test (Serotec Ltd). The acquisition of the osteogenic phenotype was confirmed by the analysis of bone-specific transcripts, by the detection of Alkaline phosphatase (ALP) activity (Sigma-Aldrich) and by mineral nodule formation. ALP activity was measured in cell lysates, with a biochemical method based on the conversion of p-Nitrophenyl Phosphate Liquid Substrate (Sigma). Mineral deposition was assessed by Alizarin red staining.

2.2. MiRNA microarray analysis

The microarray analysis was performed on samples obtained from three donors (3532, female, 34 years; 3540, male, 64 years; and 3742 female, 39 years). All MSC cultures used for the microarray analysis were able to form mineral nodules at the final time point. Total RNA was isolated using TRIzol reagent (Life Technologies), following the manufacturer's recommendations. The integrity of RNA was confirmed by agarose gel electrophoresis and its concentration determined by spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific). The miRNA expression profiling was performed using miRCURY LNA™ microRNA Array, v.11.0 — hsa, mmu & rno. Samples were labeled using the miRCURY LNA™ microRNA Power labeling kit. After hybridizing the samples in a hybridization station, arrays were scanned and analyzed with GenePix Pro v6.0 software. The statistical analysis of the differentially expressed miRNAs was performed using the paired *t*-test. The following comparisons were made: full confluence (MD4) *versus* time point 0 (MD1); mineral deposition (MM2) *versus* full confluence (MD4).

A bibliographic research of the miRNAs differentially expressed during differentiation has been performed in order to highlight their validated targets.

Table 2
Probes and primers for the detection of bone-specific genes.

Gene, reference number NCBI	Primer sequence (5′–3′)	Universal Probe Library probe
GAPDH (NM_002046.3)	F: agccacatcgctcagacac R: gcccaatacgaccaaatcc	#60
Osterix (AF477981.1)	F: catctgcctggctccttg R: caggggactggagccata	#69
ALP (NM_000478.3)	F: gggtcagctccaccacaa R: gcattggtgttgtacgtcttg	#52
COL1A1 (NM_000088.3)	F: cccctggaaagaatggagat R: aatcctcgagcaccctga	#60