RESEARCH

Study of the role of miRNA in mesenchymal stem cells isolated from osteoarthritis patients

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KEYWORDS
Mesenchymal stem cells; Microribonucleic acids; Osteoarthritis

Abstract
Objective: miRNAs act as gene silencers that are involved in the regulation of essential cell functions. miR-335 is involved in regulating cell differentiation processes in progenitor cells. Mesenchymal stem cells (MSCs) are progenitor cells of chondrocytes and osteoblasts responsible for homeostatic maintenance of cartilage and bone. The aim of this study was to determine a possible relationship between the expression of miR-335 and osteoarthritis.

Methods: MSCs obtained from the bone marrow of 3 osteoarthritic patients and 3 controls with no clinical signs of osteoarthritis or osteoporosis were cultured and phenotypically and functionally characterized in a 3-step culture. Expression levels of miR-335 and the mesoderm-specific transcript gene – MEST – that controls its expression were determined by quantitative PCR.

Results: Differences in the expression levels of miR-335 and MEST (median [interquartile range]: 1.69 [0.85–1.74], and 3.85 [3.20–5.67]) were detected between MSCs isolated from patients with osteoarthritis and controls. Although the differences detected did not reach statistical significance (P=.1), a clear trend toward lower expression of miR-335 in osteoarthritic MSCs was observed.

Conclusions: Given that miR-335 has the different genes involved in the Wnt signaling pathway as potential targets, the observed trend may help to ascertain, at least partially, some of the alterations which determine the onset or progression of osteoarthritis, and can therefore serve for the design of future therapeutic targets for the treatment of this disease.

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Estudio del papel de los miARN en células madre mesenquimales aisladas de pacientes artrósicos

Resumen
Objetivo: Los miARN actúan como silenciadores génicos que están implicados en la regulación de funciones celulares esenciales. El miR-335 participa regulando los procesos de diferenciación celular en células progenitoras. Las células madre mesenquimales (MSC) son células progenitoras de los condrocitos y osteoblastos encargados del mantenimiento homeostático del cartílago y hueso. El objetivo de este estudio era determinar una posible asociación entre la expresión de miR-335 y la enfermedad artrósica.

Metodología: Las MSC obtenidas de la médula ósea de 3 pacientes artrósicos y 3 controles sin signos clínicos de artritis ni osteoporosis se cultivaron y caracterizaron fenotípica y funcionalmente en el pase 3 de cultivo. Así mismo, mediante PCR cuantitativa se determinaron los niveles de expresión de miR-335 y del gen mesoderm-specific transcript «MEST», que controla su expresión.

Resultados: Se detectaron diferencias entre las MSC aisladas de pacientes con artritis y los controles en los niveles de expresión de miR-335 y de MEST (mediana [rango intercuartílico]: 1,69 [0,85-1,74]; 3,85 [3,20-5,67]). Aunque las diferencias detectadas no alcanzaron una significación estadística (p = 0,1), si se apreció una clara tendencia a una menor expresión de miR-335 en las MSC de pacientes artrósicos.

Conclusiones: Teniendo en cuenta que miR-335 tiene como dianas potenciales diferentes genes que participan en la vía de señalización de la Wnt, la tendencia observada podría determinar, al menos en parte, algunas de las alteraciones que determinan el inicio o progresión de la artritis, y puede, por lo tanto, servir en el diseño de futuras dianas terapéuticas para el tratamiento de esta enfermedad.

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Introduction

Osteoarthritis (or arthrosis) is the most prevalent rheumatic condition, particularly affecting individuals of advanced age. It is characterized by chronic and progressive joint damage, inflammation, pain and, in advanced stages, loss of function of the affected joints. At a tissue level, the main characteristic of osteoarthritis is sclerosis of the subchondral bone and, secondarily, progressive loss of the joint cartilage. While the alteration of articular homeostasis, resulting in a net loss of cartilage and formation of osteophytes, is concomitant with the disease, its root causes are unknown. At present, the multifactorial etiology of osteoarthritis is generally accepted, as is its association with metabolic, structural, genetic and environmental factors. Due to the evident physiological changes which take place in cartilage, for many years it has been assumed that this is the starting point of the disease. However, research conducted in recent years has highlighted the important role of the bone underlying the cartilage (subchondral bone) in the development of the disease. In this context, our group has previously described the existence of an association between a genetic polymorphism linking type X collagen and its reduced expression in mesenchymal stem cells (MSCs) with an osteoarthritic origin. These experimental findings revealed that the mechanism responsible for the correct regeneration of cartilage in osteoarthritis had its origins in both deficient formation of the extracellular matrix and possible alterations in the differentiation potential of MSCs into hypertrophic chondrocytes.

The role of MSCs in osteoarthritis is not trivial, as these are the parent cells of osteoblasts and chondrocytes, which, in turn, are responsible for synthesizing the cartilage and bone extracellular matrix and, therefore, are essential for the formation and regeneration of bone and cartilage. In light of this evidence, it is highly probable that an alteration of the differentiation process of these cells could lead to an abnormal development and tissue homeostasis, contributing to the etiopathogenesis of osteoarthritis. In this regard, our group recently described the existence of alterations in the signal pathway of Wnt/β-catenin in osteoarthritic MSCs. This pathway is essential for the control of key processes, such as cell proliferation, migration and differentiation. Gene expression is regulated by different mechanisms which allow or prevent their transcription and, ultimately, their function through the proteins they encode. One of the most recently studied regulatory mechanisms is that exerted through miRNAs. These are a family of non-coding RNAs, with an approximate size of between 18 and 24 nucleotides in length. miRNAs act as gene silencers repressing mRNA translation or inducing their degradation. Thus, depending on the biological context, they are potentially capable of modulating the signaling activity of a cell signal transduction pathway, for example, one involved in the differentiation of MSCs, by selectively suppressing or activating specific components.

Recently, due to the increasing interest in the study of miRNA regarding regulation of tissue differentiation, several miRNAs have been identified whose expression is increased during the differentiation of MSCs into different mesenchymal phenotypes. Thus, miR-26b and miR-337 regulate...
adipocytic differentiation and chondrogenesis, respectively. Several miRNAs have also been identified which act by regulating osteogenic differentiation, including miR-125b and miR-196a.\(^1\) This background indicates that miRNA expression during MSC differentiation directs the evolution of these cells toward specific lineages through a strict control of gene expression.

A key regulatory role in the biology of MSCs has recently been proposed for miR-335. Specifically, it has been reported that miR-335 (encoded by the second intron of the mesoderm-specific transcript gene [MEST]) includes the DKK1 gene, an inhibitor of the Wnt signaling pathway, among its potential targets. Given the importance of this pathway in the processes which occur during the differentiation of MSCs, our objective was to establish the possible relationship between miR-335 and osteoarthritis.

### Materials and methods

#### Selection of patients and samples

We analyzed 2 consecutive cohorts of patients undergoing total hip arthroplasty due to osteoarthritis or subcapital fracture. The diagnosis was based on the criteria of the American College of Rheumatology.\(^2\)

All patients included in the study underwent a measurement of bone mineral density of the contralateral hip and the spine including all 4 lumbar regions and measuring of the T-score <=-2.5. Subjects showing signs of osteoporosis were ruled out. All patients signed informed consent documents and the study was approved by the Ethics Committee of our center.

The **inclusion criteria** for the patients in the study were:

- Subjects undergoing hip replacement surgery due to osteoarthritis or subcapital fracture.
- Age between 60 and 70 years.
- Caucasian origin.

The **exclusion criteria** for the study were:

- Presenting concomitant disease which affected bone metabolism.
- Patients who had received steroids and/or cytostatic drugs or antiresorptive drugs in the 3 months prior to surgery.
- Presenting an additional rheumatologic involvement, such as systemic disease, spondylitis and rheumatoid arthritis.

Based on these criteria, we selected 3 patients in each cohort, considering as controls those subjects who presented subcapital fracture, without signs of osteoarthritis. On the day of the surgery we collected bone marrow aspirates and stored them in a refrigerator at 4°C until they were processed, within 18h after they were obtained.

#### Culture and cell differentiation

Each bone marrow aspirate was diluted 1:1 with Dulbecco’s Modified Eagle Medium (DMEM), and a Ficoll gradient was prepared to isolate mononuclear cells. MSCs were isolated by adherence to the culture support during expansion. The culture was performed in DMEM supplemented with 10% fetal bovine serum and antibiotics.\(^1\) All experiments in the study were conducted once cells reached 80% confluence in the third culture pass.

### Characterization of mesenchymal stem cells

The characterization of the isolated cells was carried out by analyzing the presence (CD90-PE, CD73-FITC, CD105-PE, and CD166-PE) or absence (CD45-PE) of surface markers through flow cytometry using a cytometer (Cytomics FC500, Beckman Coulter, US).

### Differentiation of mesenchymal stem cells into 3 cell lines: chondrogenic, adipogenic and osteogenic

The pluripotency of the isolated cells was examined using osteogenic, chondrogenic and adipogenic differentiation kits (Lonza LP-3002, PT-3003 and PT-3004), following the instructions provided by the manufacturer.

### Osteogenic differentiation

For osteogenic differentiation, MSCs were cultured in 24-well culture plates with MSC growth medium: DMEM +10% fetal bovine serum. When the cells reached confluence, the growth medium was replaced by another medium containing dexamethasone, L-glutamine, ascorbic acid, β-glycerophosphate and growth factors for MSCs (Lonza PT-3002). The culture was maintained for 21 days and the formation of calcium deposits was assessed by Alizarin Red S (Sigma–Aldrich, St. Louis, MO, US).

### Chondrogenic differentiation

For chondrogenic differentiation, MSCs were cultured in 24-well culture plates with MSC growth medium: DMEM +10% fetal bovine serum. When the cells reached confluence, the growth medium was replaced by another medium containing dexamethasone, ascorbic acid, transferin-selenium insulin supplement, sodium pyruvate, proline and L-glutamine and 10 ng/ml TGF-β3 (Lonza PT-3003). The culture was maintained for 28 days, changing the medium every 3 days. Chondrogenesis was assessed by staining with toluidine blue (Sigma–Aldrich, St. Louis, MO, US).

### Adipogenic differentiation

For adipogenic differentiation, MSCs were cultured in 24-well culture plates with MSC growth medium. Stimulation toward the adipogenic lineage was performed using 3 cycles of adipogenic induction and maintenance (Lonza PT-3004). After completing the 3 cycles, MSCs were cultured for 7 days in maintenance medium. Adipogenesis assessment was performed by staining with Oil Red O (Sigma–Aldrich, St. Louis, MO, US).
### Isolation of ribonucleic acid and reverse transcription

miRNA expression was determined by isolation of the total RNA from the MSCs of osteoarthritic subjects and controls, using the miRNeasy® Mini Kit protocol (Catalog No. 74104, Qiagen, Valencia, CA, US). Levels of miR-335 and MEST gene were quantified by RT-PCR using the corresponding TaqMan® (Applied Biosystems, Foster City, CA, US) gene expression assays. As endogenous controls for miRNA and MEST normalization we used RNU48 (small nucleolar RNA, C/D box 48) and glyceraldehyde-3-phosphate dehydrogenase, respectively.

### Statistical analysis

Statistical comparisons were performed using a nonparametric test (Mann-Whitney), comparing the differences between changes in expression \((2^{-\Delta\Delta CT})\) obtained (delta CT = number of cycles of the reference gene – number of cycles of problem gene). Calculations and graphs were elaborated using the GraphPad Prism® 5.01 software package. The level of statistical significance was set at a value of \(P < .05\).

### Results

#### Phenotypic and functional characterization of mesenchymal stem cells

In order to determine the uniformity of the cell populations studied, we carried out phenotypic characterization intended to ensure the purity of the isolated population by adherence and expansion in culture. All cells in the study were positive for surface markers characteristic of MSCs: CD90, CD73, CD105 and CD166. Furthermore, the isolated populations lacked hematopoietic markers like CD45.

As a final quality control, we also determined the potential of the isolated populations to differentiate into 3 mesodermal lineages (osteogenic, adipogenic and chondrogenic) under specific stimuli. All cell populations used were able to differentiate into different lineages (Fig. 1).

### Mesenchymal stem cells isolated from patients with osteoarthritis presented lower levels of miR-335 expression than controls

In order to determine whether the expression levels of miR-335 were associated with osteoarthritic disease, we analyzed the levels of differential expression of miR-335 in MSCs by quantitative PCR. The results showed that MSCs isolated from patients with osteoarthritis had lower levels of miR-335 expression (median 3.85 and interquartile range 3.20–5.67) than MSCs isolated from control subjects (median 1.69 and interquartile range 0.85–1.74), with \(n = 3\) (Fig. 2).
This reduced expression, although not statistically significant, did show a trend toward decreased expression.

Discussion

The mechanism by which MSCs are able to differentiate into and commit toward a particular lineage is not fully understood at present. However, it is known that miRNA plays a critical role in these processes through the activation and suppression of different genes at the translational level or through interactions with their target RNAs. It has been suggested that each miRNA is capable of regulating hundreds of messenger RNAs and that their action covers multiple biological functions, including embroygenesis, organogenesis, differentiation and apoptosis. Therefore, alterations in miRNA regulation and, by extension, their targets, can be essential in the development of various diseases, as described for some cases of tumorigenesis and cardiopathies or in the development of osteoarthritis.

In this context, it has recently been reported that the expression levels of miR-335 are variable during chondrogenic and osteogenic differentiation, thus probably indicating their role in controlling the transition between the phenotypes of undifferentiated and differentiated cells and in regulating cell fate in mesodermal tissue. In support of this possible role, it is known that the potential target genes of miR-335 include several transcription factors and genes related to mesodermal development.

MSCs are parent cells of chondrocytes and osteoblasts and, therefore, able to carry out the regeneration of bone and cartilage tissues. Since osteoarthritis involves a homeostatic imbalance of these tissues, the aim of our study was to establish a possible association between the expression of miR-335 in MSCs and the existence of an osteoarthritic process.

The analysis of miR-335 expression levels in MSCs indicated the possible existence of a differential expression pattern between osteoarthritic and control subjects. Although these differences were not significant, a previous study by our group, which analyzed data from a DNA microarray of the entire genome, observed a decrease in the levels of MEST gene in MSCs from osteoarthritic patients compared to control subjects, which is consistent with the current results.

Despite the fact that the expression levels of both microRNA and the MEST gene are clearly reduced, indicating a clear trend toward less expression of both in osteoarthritic MSCs, the lack of statistical significance is justified by the small sample size. Thus, this work should only be considered as a pilot study prior to the implementation of new research with a larger sample size in order to support and validate the results obtained herein and aimed at defining the exact contribution of miR-335. This study, along with observations from prior research, indicates a role of miR-335 in the pathophysiology of osteoarthritis. Specifically, we propose a key regulatory role of miR-335 in the biology of MSCs, concluding that positive regulation of miR-335 alters the repair phenotype of MSCs.

Since miRNAs act by regulating gene expression, it is logical to believe that a reduction in the expression levels of miR-335 would be detrimental to the correct regeneration of joint tissue. Therefore, it seems necessary to conduct further research in order to better understand and define the role of miR-335 and other miRNAs in the control of the mechanisms involved in tissue differentiation and regeneration in osteoarthritis. This knowledge could also contribute to the development of future therapeutic approaches.

Level of evidence

Level of evidence II.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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Conflict of interests

The authors have no conflict of interests to declare.

References