

Analysis of the Bone MicroRNome in Osteoporotic Fractures

Pablo Garmilla-Ezquerria · Carolina Sañudo ·
Jesús Delgado-Calle · María I. Pérez-Nuñez ·
Manuel Sumillera · José A. Riancho

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Abstract Osteoporosis causes important morbidity among elderly individuals. Fragility fractures, and especially hip fractures, have a particularly negative impact on the patients' quality of life. The role of epigenetic mechanisms in the pathogenesis of many disorders is increasingly recognized, yet little is known about their role in non-malignant bone disorders such as osteoporosis. The aim of this study was to explore the expression of miRNAs in patients with osteoporotic hip fractures. Trabecular bone samples were obtained from the femoral heads of patients undergoing replacement surgery for osteoporotic hip fractures and non-fracture controls with hip osteoarthritis. Levels of 760 miRNA were analyzed by real-time PCR. Thirteen miRNAs showed nominally significant ($p < 0.05$) differences between both groups. Six miRNAs (miR-187, miR-193a-3p, miR-214, miR518f, miR-636, and miR-210) were selected for the replication stage. These miRNAs were individually analyzed in a larger group of 38 bone samples. At this stage, we confirmed statistically significant differences across groups for mir-187 and miR-518f. The median relative expression levels of miR-187 were 5.3-fold higher in the non-fracture group ($p = 0.002$). On

the contrary, miR-518f was preferentially expressed in bones from osteoporotic patients (8.6-fold higher in fractures; $p = 0.046$). In this first hypothesis-free study of the bone microRNome we found two miRNAs, miR-187, and miR-518f, differentially regulated in osteoporotic bone. Further studies are needed to elucidate the mechanisms involved in the association of these miRNAs with fractures.

Keywords MicroRNA · Epigenetics · Osteoporosis · Fractures · Osteoblasts

Introduction

Osteoporosis is very common among the elderly population and causes significant morbidity, disability, and even mortality. In fact, it has been estimated that more than one third of women over 50 year suffer osteoporosis [1]. Fragility fractures are the clinically relevant consequence of osteoporosis. Among them, hip fractures have the worst consequences on the quality of life and life expectancy of the patients.

The genetic component in the pathogenesis of this disorder is clearly evidenced by its aggregation in families [2–4]. However, candidate gene and genome-wide association studies aiming to identify the loci involved have been somewhat disappointing, and the genes identified by those studies only explain a minor proportion of the genetic risk of the disease [5]. Similar findings have been reported in other complex disorders resulting from the interaction of genetic and environmental factors. In recent years, epigenetic mechanisms are emerging as important factors in the interplay between acquired and genetic risk factors. Major epigenetic mechanisms include DNA methylation, microRNAs (miRNAs) and modifications of histone tails and chromatin conformation [6–8].

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P. Garmilla-Ezquerria · C. Sañudo · J. Delgado-Calle ·
J. A. Riancho (✉)
Department of Medicine, Hospital U.M. Valdecilla, IDIVAL,
University of Cantabria, Av. Valdecilla s/n, 39008 Santander,
Spain
e-mail: rianchoj@unican.es

M. I. Pérez-Nuñez · M. Sumillera
Department of Traumatology and Orthopedic Surgery, Hospital
U.M. Valdecilla, University of Cantabria, Santander, Spain

Different from messenger RNAs, miRNAs are not translated into proteins. They are short (20–25 nucleotides) RNAs which bind to messenger RNAs bearing complementary sequences. The binding of a miRNAs to its target messenger RNA induces the degradation of the latter or halts its translation into the corresponding protein chain at the ribosomes [9–11]. Each miRNA may have several gene targets. About 2,500 miRNAs have been described, of which a few hundreds are the best characterized.

Epigenetic factors, and specifically miRNAs, are being increasingly involved in the pathogenesis of neoplastic and non-neoplastic disorders. However, little is known about their role in non-malignant disorders of the skeleton, such as osteoporosis. Therefore, the aim of this study was to explore the expression of miRNAs in patients with osteoporosis, specifically, with fragility hip fractures, which represent the most ominous consequence of osteoporosis.

Materials and Methods

Bone Samples

Bone samples were obtained from the femoral heads of patients undergoing hip replacement because of hip fracture. As a control group (hereafter referred to as non-fracture), we chose patients with severe hip osteoarthritis, without history of osteoporotic fractures.

In the discovery stage the study group included eight women with hip fractures (age 81 ± 4 year) and 8 non-fractured controls with osteoarthritis (age 75 ± 8 year). In the replication stage 19 patients with fractures and 19 with osteoarthritis were included. Patients with high-energy fractures, cancer, other severe chronic disorders, secondary osteoporosis, secondary osteoarthritis, or taking drugs known to affect bone metabolism were excluded. Accordingly to the epidemiology of these disorders, the group of patients with fractures had a slightly higher average age and frequency of diabetes (Table 1). Trabecular bone cylinders of the central part of the femoral head were obtained with a trephine. Both edges were removed in order to avoid both the fracture or surgically cut regions, as well as the subchondral region. The remaining fragments were cut in small fragments, washed extensively in phosphate-buffered saline and either snap-frozen in liquid nitrogen and stored at -70 °C or used to set up osteoblast cultures by the primary explant technique [12]. In brief, bone fragments were seeded into T-75 plastic flasks containing Dulbeccó's modified medium with antibiotics and 10 % fetal bovine serum. This allowed osteoblastic precursor cells to migrate from the fragments and proliferate. After confluency, cells were trypsinized and

Table 1 Patient characteristics

Experimental stage	Sex, women (<i>n</i>)	Age (mean, SD and range)	Diabetes mellitus (<i>n</i>)
Discovery			
Fractures (<i>n</i> = 8)	8	81 ± 4 (76–85)	3
Non-fractures (<i>n</i> = 8)	8	75 ± 9 (58–84)	1
Replication			
Fractures (<i>n</i> = 19)	18	77 ± 4 (71–84)	5
Non-fractures (<i>n</i> = 19)	18	73 ± 3 (70–81)	2
Osteoblast cultures			
Fractures (<i>n</i> = 8)	8	79 ± 4 (74–84)	2
Non-fractures (<i>n</i> = 8)	8	77 ± 6 (62–84)	2

cultured in the same medium. When they were confluent again, medium was aspirated and the cells were cultured in serum-free medium containing 0.1 % bovine serum albumin for 48 h before RNA extraction.

miRNA Extraction and Quantitation

Total RNA was extracted from osteoblast cultures using Trizol, following manufacturer's recommendations (Invitrogen-Life Technologies, Foster City, CA, USA). To extract RNA from bone, frozen samples kept at -70 °C were again immersed in liquid nitrogen and then quickly crashed with a hammer, transferred into ice-cold Trizol and homogenized with a tissue disruptor. RNA extraction was continued as mentioned above. After quantification and integrity checking by gel electrophoresis, RNA from each individual was then reverse-transcribed using MultiScribe RT and one of two pools of primers (Megaplex RT primers pool A and pool B; Life Technologies), each including primers for 384 miRNAs, in an ABI9700 thermal cycler. cDNA was then pre-amplified using Taqman preamp master mix (Life Technologies) and the corresponding primer pools (Megaplex Preamp primers pool A and pool B; Life Technologies), with the following protocol: 95 °C \times 10 min; 55 °C \times 2 min; 72 °C \times 2 min; followed by 12 cycles of 95 °C \times 15 s and 60 °C \times 4 min; and final inactivation at 99.9 °C \times 10 min. The product was diluted in nuclease-free water and each miRNA was measured by real-time quantitative PCR (see below). Samples from different groups were distributed equally in

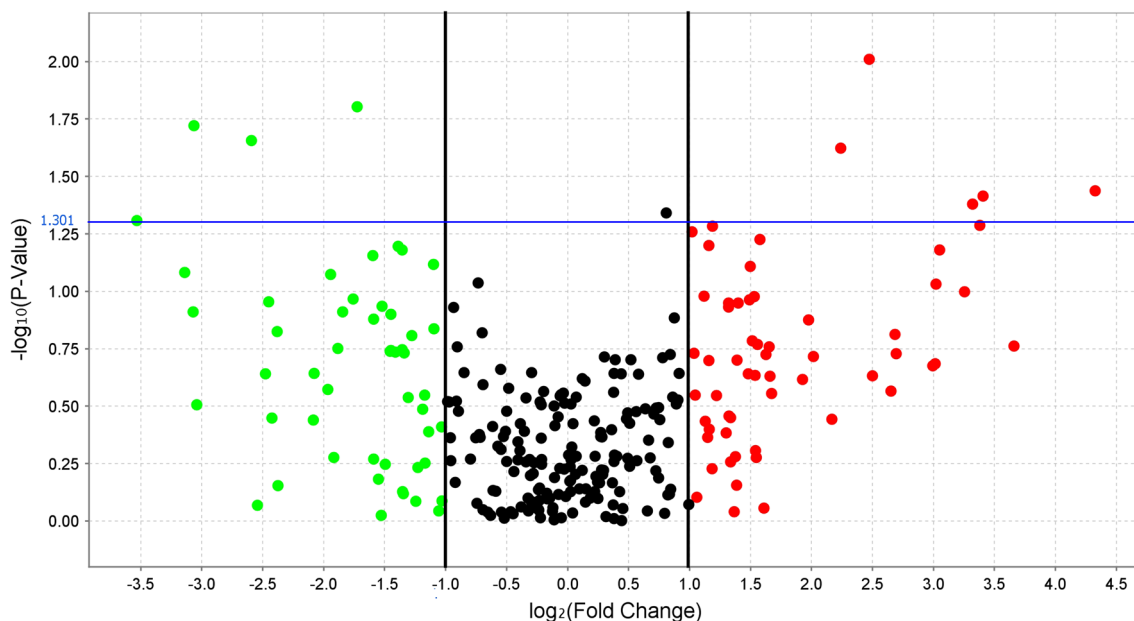


Fig. 1 Volcano plot of the miRNAs included in pool A set analyzed in eight patients with fractures and eight without fractures. It shows, for each miRNA, the fold-change (as the log₂-transformed ratio of the

expression in osteoporosis and in osteoarthritis bones) and the corresponding *p* value (as $-\log_{10}$ values)

each experimental run in order to minimize experimental batch bias.

Discovery Stage

The level of 760 miRNAs was determined by real-time quantitative PCR using miniarrays (Taqman array human miRNA A + B cards v3, Life Technologies). These comprise two 384-well plates (pool A and pool B sets) including assays for 380 target miRNAs and 4 miRNAs that can be used as controls. The pool A set includes some of the best characterized and most abundant miRNAs. The complete list of miRNAs is available from the supplier (http://tools.lifetechnologies.com/content/sfs/brochures/cms_054742.pdf) and also shown in the supplementary online materials (Tables S1 and S2). The reaction set up and the amplification protocol followed the manufacturer's instructions. The cycle threshold (Ct, the PCR cycle at which the fluorescence signal threshold is achieved) was estimated for each miRNA. In order to find out the best miRNAs for normalization, we used the NormFinder and GeNorm packages, implemented in Genex software (MultiD AB, Goteborg, Sweden). These programs identified miR-222 and let-7b as ideal for normalization due to its stable expression across samples and groups. The level of each miRNA was then estimated by relating, for each sample, the Ct of the test miRNA and the average Ct of the normalization miRNAs, using the $2^{-\Delta Ct}$ method, where ΔCt is the difference between the test miRNA Ct and the Ct for the normalizing

miRNAs. A global normalization procedure (including all the targets in each pool set) was then used to account for the between-array variability.

Replication Stage

Selected miRNAs showing statistically significant differences in the discovery phase were replicated by analyzing them in a larger group of samples. Patients were selected according to the same criteria. RNA was isolated as in the discovery phase, mentioned above. After reverse transcription, the level of each miRNA was determined by real-time PCR using individual Taqman assays following manufacturer's instructions (Life Technologies) in duplicate wells and normalized according to the expression of let-7b and miR-222 using the $2^{-\Delta Ct}$ method. Between-group differences were analyzed by the nonparametric Mann–Whitney *U* test. All comparisons were two-tailed and *p* values less than 0.05 were regarded as statistically significant.

Results

Discovery Stage

Among the 380 miRNAs included in the “pool A” set, 128 (34 %) were detected (i.e., Ct < 35) in all samples, and 74 % were detected in at least 12 (75 %) of the 16 bone

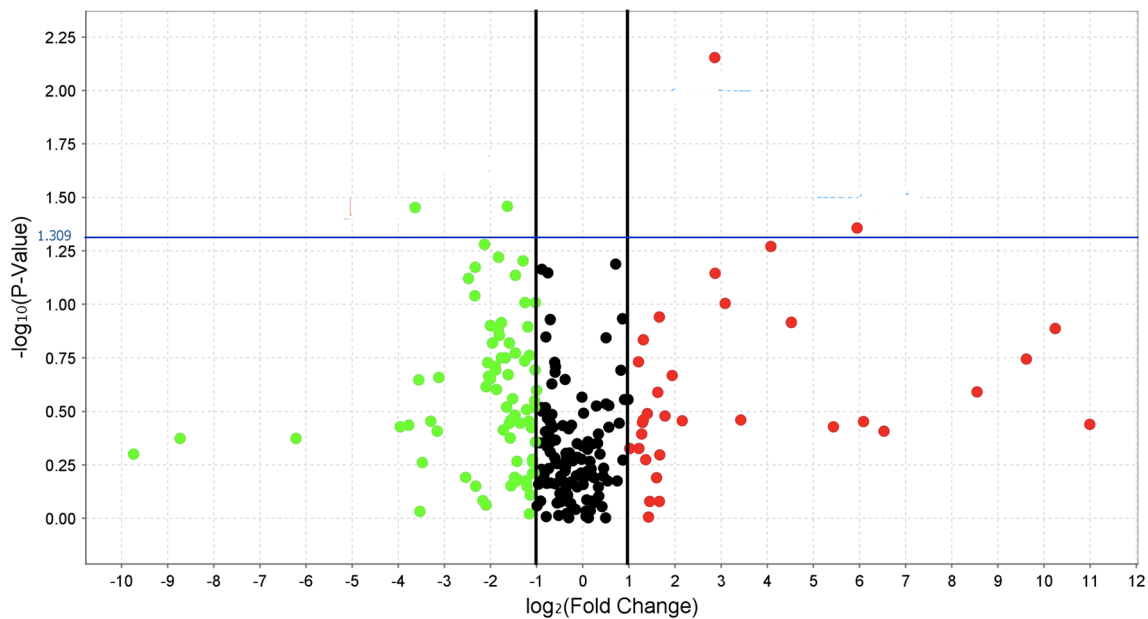


Fig. 2 Volcano plot of the miRNAs included in the pool B set analyzed in eight patients with fractures and eight without fractures. It shows, for each miRNA, the fold-change (as the \log_2 -transformed

ratio of the expression in osteoporosis and in osteoarthritis bones) and the corresponding p value (as $-\log_{10}$ values)

samples. As expected, miRNAs included in “pool B” set were detected less frequently than those in “pool A”. Only 104 miRNAs (27 %) were detectable in all 16 samples, whereas 51 % were detectable in at least 12 bone samples.

The comparison of the expression levels of miRNAs in osteoporotic and control bones is shown in Figs. 1 and 2. Overall, 13 miRNAs showed nominally significant ($p < 0.05$) differences between both groups; seven were more abundant in bones from osteoporotic patients, whereas six were more abundant in the non-fracture bone samples (Table 2).

Replication Stage

Five miRNAs with statistically significant differences in expression in the discovery stage were selected for the replication stage, after taking into consideration p values, fold-changes, and the consistency of amplification. They included miR-187, miR-193a-3p, miR-214, miR518f, and miR-636. These miRNA were analyzed in a larger group, including samples from 19 patients with osteoporotic hip fractures and 19 non-fractured individuals. Additionally, we also explored miR-210, which expression in the discovery stage was close to statistical significance ($p = 0.06$) and was reported to be associated with osteoblast activity in human bone samples in a recent study [13].

At this stage, we confirmed statistically significant differences across groups for miR-187 and miR-518f. miR-187

was detected (i.e., $Ct < 35$) in 90 % of non-fractured samples, but only in 74 % of osteoporotic bone samples. The median relative expression levels (normalized to the mean Let-7b and miR-222 levels) were 3.7×10^{-5} and 1.9×10^{-4} in the osteoporosis and non-fracture group, respectively (Mann–Whitney U test $p = 0.002$) (Fig. 3). On the contrary, miR-518f was preferentially expressed in bones from osteoporotic patients ($p = 0.046$). It was detected in 74 % of fracture samples and only in 37 % of non-fracture samples, with median normalized expression levels of 1.8×10^{-4} and 2.1×10^{-5} , respectively ($p = 0.046$; Fig. 3). Similar results were observed if the samples from patients with diabetes were excluded from the analysis, with slightly higher p values due to the reduced sample size ($p = 0.006$ and 0.07 for the differences of miR-187 and miR-518f between patient groups, respectively). We did not confirm the differences across groups regarding miR-636, miR-210, miR-214, and miR193a-3p (Fig. 4).

miRNA Analysis in Osteoblast Cultures

We set up primary osteoblast cultures grown from eight bones from patients with osteoporosis and 8 non-fracture samples and analyzed the presence of miR-187 and miR-518f. These miRNAs were detectable ($Ct < 35$) in 60 and 55 % of those samples, respectively. No statistically significant between-group differences were observed ($p = 0.9$ and 0.2 for miR-187 and miR-518f, respectively).

Table 2 Micro-RNAs showing significant differences between osteoporosis and non-fracture individuals at the discovery stage

MicroARN	Fold-change	<i>p</i>
Let-7i	0.1	0.035
miR-135a	5.7	0.010
miR-137	10.6	0.038
miR-181a-2	0.3	0.035
miR-187	0.1	0.049
miR-193a-3p	0.2	0.022
miR-214	0.3	0.016
miR-330-5p	0.1	0.019
miR-518f	19.7	0.037
miR-519d	9.8	0.042
miR-524	59.7	0.044
miR-636	4.9	0.024
miR-643	7.5	0.007

The fold-changes (fracture vs. non-fracture) and *p* values are shown

Discussion

Although all the cells in our body share the same genotype, only a defined set of genes are expressed in a given tissue at a certain time. This allows each individual cell, and consequently the organism, to adapt to the changing environmental conditions, and biological needs. Epigenetic mechanisms are responsible for that adaptation. Whereas the general on–off switching of genes appear to be largely related to specific changes in the methylation of cytosines in DNA, miRNAs may be more importantly involved in the fine and rapid tuning of the protein synthesis. Thus, miRNA-mediated mechanisms are attractive candidates to be explored as potential regulators of bone remodeling (a process in which osteoclasts and osteoblasts act in an ordered process, site-coupled and lag-synchronized in time).

In the present study we found, after the discovery and the replication stages, two miRNAs, miR-518f, and miR-

187, differentially expressed in osteoporosis and osteoarthritis. According to prediction algorithms (microRNA.org, mirbase.org, targetscan.org), miR-518f targets several genes involved in skeletal homeostasis, such as IGFBP1, and several factors related to the Wnt pathway (DKK1, WISP1, or CTNNBIP1). The canonical Wnt pathway is a central stimulator of osteoblast differentiation [14, 15]. In fact, mutations of genes involved in the pathway have profound consequences on bone mass [16–18]. We have shown that β -catenin nuclear levels are diminished in primary osteoblasts from patients with osteoporotic fractures, in comparison with those from patients with osteoarthritis [19]. In keeping with this, the upregulation of miR-518 in osteoporotic bone could contribute to the down-regulation of the Wnt pathway. On the other hand, miR-187 was down-regulated in patients with osteoporotic fractures. Although miR-187 regulates the expression of some genes, such as those encoding the proinflammatory cytokines IL-6 and TNF, which may modulate the activity of skeletal cells [20] (Fig. 5), its actual role in bone physiology is still unclear. The differences in miRNA levels found in bone tissue were not replicated in bone cell cultures. This suggests that those differences are not the result of intrinsic genetic differences, but the consequence of adaptative changes or cellular interactions in bone tissue not present in cultured cells.

A number of investigators have used in vitro culture systems and animal models trying to elucidate the role of some miRNAs in the regulation of bone cell activity [6, 10, 21–29]. However, only scarce data are available about the involvement of miRNAs in common human skeletal disorders, such as osteoporosis and osteoarthritis. Li et al. [30] first reported about a role of miRNAs in osteoporosis. They found that some rare cases of osteoporosis in young individuals were due to a mutation that blocked the expression of miR-2861, but they did not find the mutation among adults with osteoporosis. Wang et al. [31] identified miR-214 as a miRNA regulating the levels of ATF4, a transcription factor involved in osteoblast differentiation, and

Fig. 3 Expression levels of *miR-187* and *miR-518f* at the replication stage in 19 patients with fractures (*Fr*) and 19 without fractures (*Non-Fr*). The values represent the relative levels normalized for the levels of the control miRNAs (let-7b and miR-222)

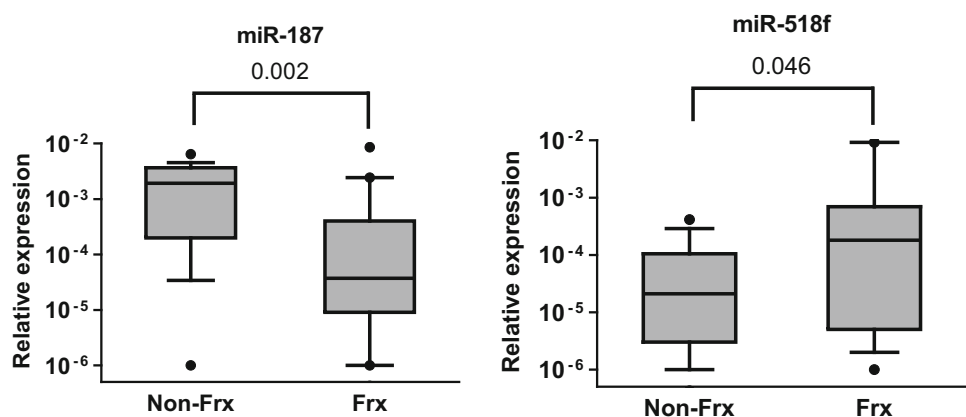


Fig. 4 Expression levels of miR-636, miR-210, miR-214, and miR193a-3p at the replication stage in 19 patients with fractures (*Fr*) and 19 without fractures (*non-Fr*). The values represent the relative levels normalized for the levels of the control miRNAs (let-7b and miR-222)

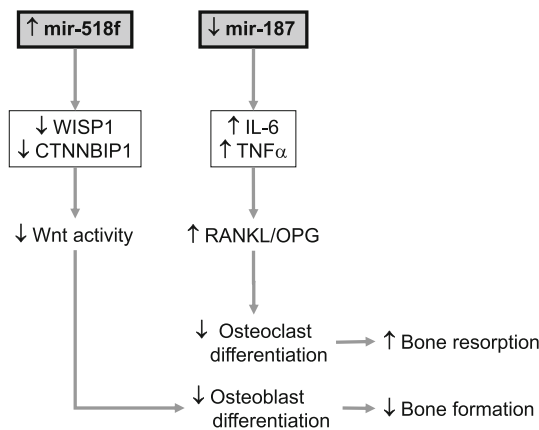
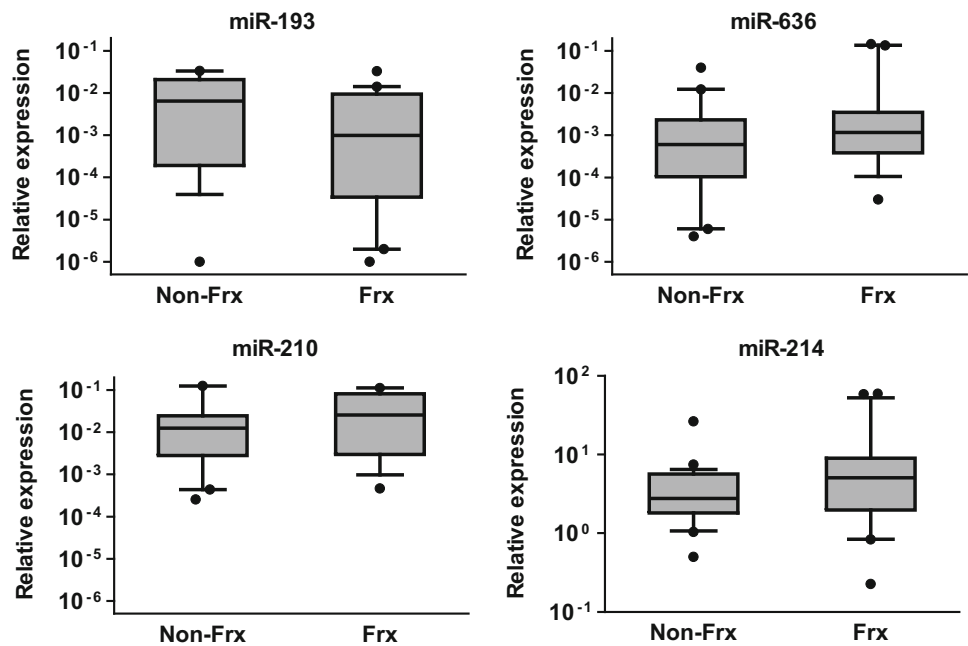


Fig. 5 Hypothetical relationships between the observed differences in miRNA expression and bone homeostasis. *WISP1* WNT1-inducible-signaling pathway protein 1, *CTNNBIP1* beta-catenin-interacting protein 1, *IL* interleukin, *TNF* tumor necrosis factor

reported that the levels of miR-214 in bone samples were negatively correlated with the expression of some genes associated with osteoblastic bone formation, such as osteocalcin and alkaline phosphatase. Thus, it could be speculated that an increased expression of miR-214 was involved in osteoporosis. However, we did not confirm this concept in our study. Indeed, in the discovery phase miR-214 levels were somewhat lower in bones from patients with fractures than in control non-fractured bones, and no statistically significant differences were found in the larger group of samples included in the replication phase.

Seeliger et al. [32] recently reported on the miRNA levels in bone samples from patients with hip fractures.

Similarly to our study, they did not replicate the previously reported association of miR-214 with osteoporosis. On the other hand, they found six miRNAs upregulated in patients with osteoporosis: miR-21, miR-23a, mir-24, mir-25, mir-100, and miR-125b. However, we did not replicate those results in our study. The reasons for these discrepant results are unknown, but may include the different procedures to select the miRNAs, the analytical techniques, as well as the type of fractures and the sex distribution of the individuals.

To our knowledge, this is the first study aimed to analyze bone miRNAs comprehensively, with a hypothesis-free approach. Nevertheless, our results, as well as others previously published, must be interpreted in view of their limitations. The sample size of the present investigations was relatively small, similar to other previously published, particularly during the discovery stage. Therefore, we were only able to pinpoint those miRNAs showing the most marked differences across groups. Other miRNAs with more subtle differences in expression may have gone unrecognized. In order to avoid further loss of power, we did not adjust the significance threshold for multiple comparisons. Nevertheless, the rate of false positive results was controlled by confirming the suggestive signals at the replication stage. Also, due to practical reasons, only the most promising signals were put forward the replication phase. Given the practical and ethical difficulties to obtain bone samples from true healthy controls, we used samples from patients undergoing hip replacement surgery for osteoarthritis as a comparison group. Although we removed bone tissue close to the subchondral region, we cannot exclude the possibility of changes in the miRNA

expression pattern distant to the osteoarthritis focus. Therefore, it is unclear if the differences between the fracture and non-fracture groups are driven by deviations from normal bone in any or both groups. We made an effort to exclude patients with severe diseases that could confound the results. However, there were some small differences in the mean age and the frequency of diabetes mellitus between the fracture and non-fracture groups. Those differences are reflection of the different epidemiology of these disorders and they seem unlikely to confound the results. In fact, we did not find clear age-related differences in miRNA expression (not shown). Likewise, similar results were obtained if the samples of patients with diabetes were excluded from the replication analysis. However, the small sample size limits the power of statistical procedures, and particularly of multivariate adjustments. Also, in view of our results it cannot be established whether the observed differences in miRNA expression are the cause or the consequence of the disease.

Given the small number of studies exploring the expression of miRNAs in human bones and the lack of replication of differentially expressed miRNAs across studies, much more research is needed to elucidate their role in the pathogenesis of skeletal disorders. It may be particularly important to perform studies with enough statistical power. Nevertheless, given the wide interindividual variations observed, rather large numbers of samples will be necessary. This may be difficult because of cost reasons and also the complexities inherent to obtaining bone tissue samples. Establishing large collaborative consortia on bone epigenetics, following the paths set for the genomic association studies, may help to accomplish this goal [5, 33]. Also, from the experience accumulated by comparing the results of genome-wide association studies and candidate gene studies, an unbiased “miRNome-wide” approach may be preferable. Mini-array technologies, as used in the present study, and novel technologies, including whole transcriptome analysis, may result particularly suitable.

In conclusion, in this first hypothesis-free study of the bone microRNome we found two miRNAs, miR-187 and miR-518f, differentially regulated in osteoporotic bone in comparison with non-fracture bones from patients with osteoarthritis. Further studies are needed to elucidate the mechanisms involved in the association of these miRNAs with fractures.

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Conflict of interest Pablo Garmilla-Ezquerria, Carolina Sañudo, Jesús Delgado-Calle, María I. Perez-Nuñez, Manuel Sumillera, and José A. Riancho declare that they do not have conflicts of interest.

Human and Animal Rights and Informed Consent The study was approved by the Institution’s Ethical Committee and informed consent was obtained from the participants.

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