Author’s response to reviews

Title: Signature of microRNA expression during osteogenic differentiation of bone marrow MSCs reveals a putative role of miR-335-5p in osteoarthritis

Authors:

Pilar Tornero-Esteban (mptornero@gmail.com)
Luis Rodríguez-Rodríguez (lrodriguezr hcsc@salud.madrid.org)
Lydia Abásolo (lydia.abasolo@salud.madrid.org)
María Tomé (mtome@cnb.csic.es)
Pedro López-Romero (plromero1@gmail.com)
Eva Herranz (evaherranzdilp@hotmail.com)
Manuel A González (manglez75@hotmail.com)
Fernando Marco (fmarco@med.ucm.es)
Enrique Moro (enrmoro@hotmail.com)
Benjamín Fernández-Gutiérrez (benjamin.fernandez@salud.madrid.org)
José Ramón Lamas (jrlamas@gmail.com)

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Author’s response to reviews: see over
Dear Editor.

Taking in consideration the comments made by reviewers of the Arthritis Research and Therapy, this manuscript has been evaluated with some major concerns, however we firmly believe that questions posed by the reviewers can be reasonably answered. Therefore, It would be very much appreciated if you would reconsider our paper for publication in **BMC Musculoskeletal Disorders**.

In response to the comments by previous reviewers, we provide the following explanations.

Best regards!

Reviewer number: 1

Referee's comments to the author(s)

This study performed miRNA expression profiling in BM-MSCs from OA patients, and examined their role during osteogenic differentiation. The authors first compared miRNA expression profiling during osteogenic differentiation of bone marrow MSCs from OA and healthy donors, and found that 246 miRNAs were differentially expressed between OA and non-OA BM-MSC samples. Among them, hsa-miR-210 and hsa-miR-335-5p out of 21 used for validation showed a significant downregulated expression during induced osteogenesis. miR-335-5p downregulation in OA-MSCs, as well as the host coding gene, MEST, were also detected, while the target genes DKK1 and SFRP1 were similar both in undifferentiated OA- or Control-MSCs. This study is well-written and meticulously discussed; however, this reviewer has several major concerns.

1. Although study starts expression profiling using comprehensive methods and found that 246 miRNAs were differentially expressed between OA and non-OA BM-MSC samples. However, selection criteria thereafter remain unclear. Although the authors state that 21 miRNAs were selected according to their lower q values (Table 1), this is not convincing and the real data for the selection should be shown. Furthermore, method of selection of hsa-miR-210 and hsa-miR-335-5p out of 21 is also unclear.

Selection criteria of miRNAs was made according to those miRNAs that showed the more statistical significant expression during osteogenesis after multiple test correction during. Given that two platforms were used for those miRNAs with undefined 5p or 3p nomenclature, both miRNAs were included. In the same way selection of hsa-miR-210 and hsa-miR-335-5p out of 21 was according to the same criteria.

2. Figure 2: Likewise, the difference of downregulation of expression during osteogenesis between hsa-miR-210 and hsa-miR-335-5p is unclear. More
detailed comparison method between the two miRNAs should be shown. Otherwise, neither selection of miR-335-5p nor abandonment of miR-210 in persuading. What is the reason the miRNA should be related to the Wnt signaling?

Probably this figure was misunderstood, both hsa-miR-210 and hsa-miR-335-5p showed a downregulated expression during osteogenesis progression. The reason to select miR-335-5p was because this miRNA has many Wnt putative targets and thus the study was focused only in this miRNA. Obviously further studies about the role of miR-210 would be interesting but beyond the scope of our study. The relationship of miR-335 and Wnt was interesting given that this pathway regulates osteogenic differentiation of MSCs. Involvement of Wnt pathway has been extensively described in literature as well as the correlation of Wnt activity with increased osteogenesis. The fact that during osteogenic stimulation no major differences were observed, after histochemical staining, indicates that this pathway was equally active in OA and controls, and that functional differences, if any, should be minimal or collaterally compensated by the regulation by other pathways.

3. It seems that miR-335-5p was selected solely based on a previous report showing that the increased expression levels of miR-335-5p regulate bone thus activating Wnt signaling [ref. 17]. In this sense, this manuscript lacks novelty.

We do not agree with this assessment because our experimental system was performed using real OA pathologic samples, thus we do not only describe the putative role of miRNA-335 but also its possible implication in this disease.

4. Figure 4: The expression of target genes DKK1 and SFRP1 of miR-335-5p were similar both in undifferentiated OA- or Control-MSCs. Besides the expression, how about the regulation of function of the Wnt signals by miR-335-5p? Functional study like TOPFlash would be needed to strengthen the manuscript.

This effect can be explained by the effect of other regulatory mechanisms not detected that might influence the expression of both genes. Although miR-335 inhibit the expression in vitro of DKK1 and or SFRP, in vivo this effect was not observed. Although we agree that functional studies like TOPFlash would be interesting to strengthen the manuscript limited availability of MSCs prevented this type of studies. given the nature of the samples studied It was difficult for us to obtain the additional results requested. Keep in mind that our samples were obtained from MSCs of patients suffering OA, these samples are relatively available due to the routinely surgery of these patients; however, the most limiting issue was to obtain good control samples. Moreover, after isolation,
characterization and testing the phenotypic differentiation and multilineage differentiation potential of cells, availability of biological material (cell cultures) for further studies was nearly impossible without excessive cell passages. These methodological approaches can only be overcame after processing new samples for these specific experiments.
Reviewer number: 2

Referee's comments to the author(s)

“The significance of the study in regard to MSC biology and osteoarthritis pathogenesis is unclear. The question whether there is a dysfunction of BM MSC from human OA patients has been addressed in several studies with variable results and conclusions. The present study is deficient in not having analyzed for differences in the proliferative and differentiation potential of their set of OA BM MSCs. Although differentiation assays were performed, there is no analysis for differences between OA and osteoporotic samples. Baseline differences in miRNA expression between OA and the control group would also be of interest but this had not been analyzed. There is also no compelling rationale for the selection of the differentially expressed miRNAs that were analyzed in more detail.”

Even though cell proliferation and survival have not been measured in our experiments, in our experience growth rate and survival are subject specific parameters, more than related with OA pathology; in fact, although generally we observed in older individuals a slower growth rate this is not always consistent. Moreover, an aspect to consider is that excessive cell passages greatly affects cell survival. This is the reason why some experiments were not fully validated by several techniques due to the low number of cells available at lower passages. Difficulties to control both parameters, in particular the cell senescence, were issues precluding some of the experimental validations suggested.

The rationale for the selection of the differentially expressed miRNAs was described above. MiR-335-5p was selected because this miRNA has many Wnt putative targets. This pathway was closely implicated in differentiation mechanisms of MSCs. Thus we focused the study only in this miRNA. After an intensive screening and further validation it was interesting that downregulation of miR-335 was consistent thus indicating a possible role of this miRNA in pathology without excluding any other role of miR-210. Although the exact mechanism was not elucidated, we considered that this work provide interesting keys for further studies.