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Research Article

Sex dimorphic response to osteocyte miR21 deletion in murine calvaria bone as determined by RNAseq analysis

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Abstract

Low levels of microRNA (miR) 21 may explain the higher osteocyte apoptosis with Cx43-deficient and aged female mice. However, miR21 exerts a sex-divergent role in osteocytes, regulating bone mass and architecture through non-cell autonomous effects on osteoblasts and osteoclasts, via sex-specific regulation of osteocyte cytokine production. miR21 deficiency improves bone strength in females, and, to a higher extent, in male miR21-deficient mice. To understand the molecular basis for the effects of miR21 deletion, mRNA was isolated from miR21$^{fl/fl}$ (controls) or miR21-deficient (by deletion in cells expressing Cre recombinase under the control of the 8 kb fragment of the DMP1 promoter: miR21$^{\Delta Ot}$ mice). miR21 was 50% lower in miR21$^{\Delta Ot}$ whole calvaria bone compared to control mice of the corresponding sex. RNAseq was performed in 4 samples/sex and genotype. There were 152 genes with $<.05$ *P*-value and >1 absolute log2 fold change in the male data analysis, and expression of most genes was higher in the miR21$^{fl/fl}$ group. Two of the genes, Actn3 and Myh4, had a false discovery rate < 0.1. Gene enrichment analysis of significant genes on both KEGG pathways and gene ontology (GO) gene sets shows that the significant genes were enriched in muscle contraction. Some muscle-related genes like Actn3 were included in multiple significant pathways. For females, only 65 genes had *P*-value $<.05$ and >1 absolute log2 fold change. Yet, no significant KEGG or GO pathways, including ≥ 5 significant genes, were seen, and no overlap of significant genes was found between male and female samples. Therefore, deletion of miR21 has a stronger effect on male transcriptome in calvaria, compared to females. Further, no enrichment of any pathway was detected in female samples. Thus, either there are no differences between 2 groups in female or the effect size is small, and a larger sample size is needed to uncover miR21-dependent differences.

Lay Summary

Small RNAs, name microRNAs or miRs, modulate the expression of genes by targeting messenger RNA leading to its degradation and the suppression of protein synthesis. We have previously shown that removal of miR21from murine osteocytes, the cells embedded in the mineralized bone matrix, results in changes in bone at the structural and biomechanical level, as well as in the number and activity of bone cells. Importantly, these effects differ between males and females. We now show that the genes regulated by the removal of the miR are also different in bones from males and females at 4 mo of age. As expected, the expression of most genes was higher in the control mice expressing miR21, compared to miR21-deficient animals. However, although 152 genes were significantly different in bones from male mice, only 65 were different in females, and there was no overlap between the genes changed in males and females. This study adds to the evidence that sexual dimorphism plays a role in skeletal development and growth.

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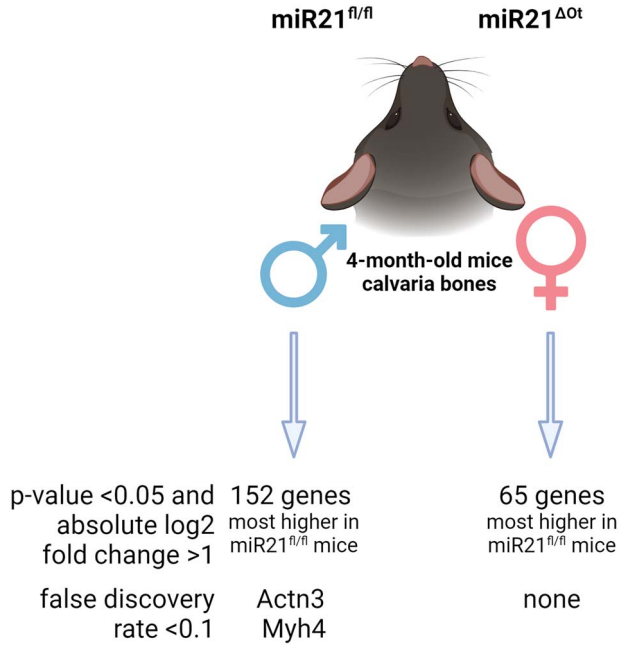
Graphical Abstract



Introduction

MicroRNAs (miRs) are 18-22-nucleotide long noncoding RNAs that negatively regulate gene expression.[1] Changes in miR levels have been associated with phenotypic changes with aging and diseases, including osteoporosis.[2] Among the most commonly studied miRs, miR21, a pro-survival and oncogenic miR, has been shown to directly regulate osteoblast and osteoclast differentiation and function.[3] In addition, we have shown that miR21 is reduced with Cx43 deficiency and aging in female mice, and that its deletion from osteocytic cells results in a sex-dimorphic effect on the skeleton.[4-6]

In particular, we showed that expression of the 8kbDMP1-Cre leads to significant decrease in miR21 levels in calvaria bones from miR21$^{\Delta Ot}$ female and male mice.[5] These miR21-deficient mice exhibit a skeletal phenotype that depends on the sex of the animals, with lower osteoblast- and osteoclast-related measurements in females and higher in males, resulting in no change in bone mass in females and higher bone mass in the males. In addition, femoral bone mechanical strength was higher in both sexes, albeit the changes were more pronounced in male mice. Thus, our published studies suggest that miR21 regulation in osteocytes alters bone metabolism and geometry through paracrine actions on osteoblasts and osteoclasts. At the same time, removal of osteocytic miR21 enhances bone mechanical properties in a sex-independent manner. Changes depending on the sex of the animals were also observed on the levels of circulating chemokines and on the activation of intracellular kinases. However, the transcriptional changes leading to the sex-specific effects were not reported.

We now report that deletion of miR21 in cells expressing the 8 kb fragment of the DMP1 promoter results in transcriptional changes in calvaria bones, and that these changes differ between female and male mice. Thus, deletion of the miR has a more profound effect on cells from male mice, and, although there are some significant changes in samples from female mice, they are not enriched in any of the reported pathways.

Materials and methods

Mice

The mouse strain engineered with loxP cassettes on both sides of the mmu-miR-21 genomic locus (named miR21$^{fl/f}$) was bred with mice harboring DMP1-8 kb-Cre in order to obtain miR21$^{f/f}$; DMP1-8 kb-Cre mice (named miR21$^{\Delta Ot}$) lacking the miR21 gene preferentially in osteocytes (Figure 1), as previously reported by us.[4,5] Mice were maintained in a C57BL/6 background, and miR21$^{fl/fl}$ littermates were used as controls. Male and female mice were euthanized at 16 wk of age. All animal procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and were carried out in accordance with institutional guidelines. Five same sex mice were housed in each cage, with water and food at libitum.

mRNA isolation and RNAseq

mRNA was isolated from calvaria bones of 4-mo-old male and female mice expressing (miR21$^{fl/fl}$ controls) or with reduced miR21 levels (miR21$^{\Delta Ot}$ experimental mice). Four samples/sex and genotype were submitted for RNA sequencing, each corresponding to an individual mouse. Bones were removed from the mice, cleaned of soft tissue using sterile gauze, cut into small pieces, and grinded using a Polytron in the presence of Trizol (Invitrogen), without removing the sutures,[5] followed by purification using QIAseq® FastSelectTM RNA Removal Kit. The 260/280 ratio ranged from 1.67 to 1.98 for all samples.

RNAseq reads were aligned to GRCm38 using HISAT and read counts for each gene were extracted with featureCounts. RNAseq reads were trimmed using Trimmomatic version 0.36 with the parameters "2:20:5 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:17" to remove adapter sequences and perform quality trimming.[7] The resulting reads were mapped against GRCm38 using HISAT2 version 2.1.0 with default parameters.[8] HISAT uses Bowtie2, which is based on the Burrows–Wheeler transform algorithm, for sequence

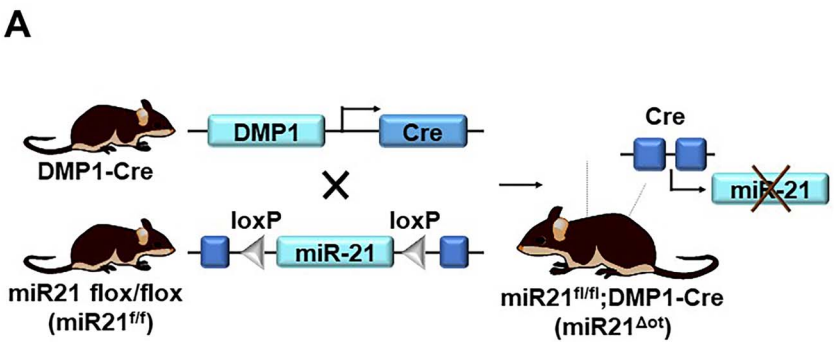


Figure 1. Generation of male and female miR21-deficient mice (miR21$^{\Delta Ot}$). Scheme illustrating generation of mouse model with osteocyte-targeted miR21 deletion. Cre, cre recombinase; DMP1, dentin matrix protein 1.

alignment and allows for mapping across exon junctions.[9] Read counts for each gene were created using featureCounts[10] from the Subread package version 1.5.3 with the parameters "-O -M –primary –largestOverlap -s 2 -B" and GENCODE m21 as the annotation.[11]

Statistical analyses

For data analyses, first, principal component analysis was performed to check data quality and detect outliers. DESeq2 was employed to detect differentially expressed genes.[12] Within DESeq2, read counts data were normalized with median of ratios, and differentially expressed genes (DEGs) were detected after independent filtering. Gene set enrichment analyses based on significant genes were performed on both Gene Ontology (GO)[13] and KEGG[14] pathways using R package clusterProfiler.[15]

Results

Calvaria bones isolated from 4-mo-old mice were used for these studies, based on our previous studies in which we showed differences in gene expression with miR-21 deficiency in male and female mice,[5] and demonstrated the effects of miR-21 deletion ex vivo on osteocytic lacuna occupancy, in the mRNA levels of FoxO3, p27, and GADD153, and in PTEN protein levels.[4] Samples from 4 mice/genotype and sex were initially processed. The read counts of genes extracted with featureCounts are included in Table S1. During quality control, sample 173 (female miR21$^{\Delta Ot}$) was removed from the analysis as an extreme outlier (Figure S1). The sex of the animals was confirmed by the expression of Uty, Eif2s3y, and Ddx3y genes, all encoded in the Y chromosome only in samples from male mice (Figure S2). On the other hand, only samples from female mice expressed Xist, a gene expressed cells with 2 X chromosomes involved in X chromosome inactivation.

The remaining samples were used to detect DEGs between groups. There were 152 genes with *P*-value less than .05 and absolute log2 fold change larger than 1 in the analysis of male data (Figure 2A). Most of these genes were expressed at a higher level in the miR21$^{fl/fl}$ group. With the 152 significant genes, the 8 male samples could be hierarchically clustered into miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ groups (Figure 3A, Table S2). Two of the genes, Actn3 and Myh4, had a false discovery rate less than 0.1 (Table S2). Gene set enrichment analysis of significant genes on both KEGG pathways and GO gene sets shows that the significant genes were enriched in muscle contraction. Some muscle-related genes like Actn3 were included in multiple significant pathways (Figure 4).

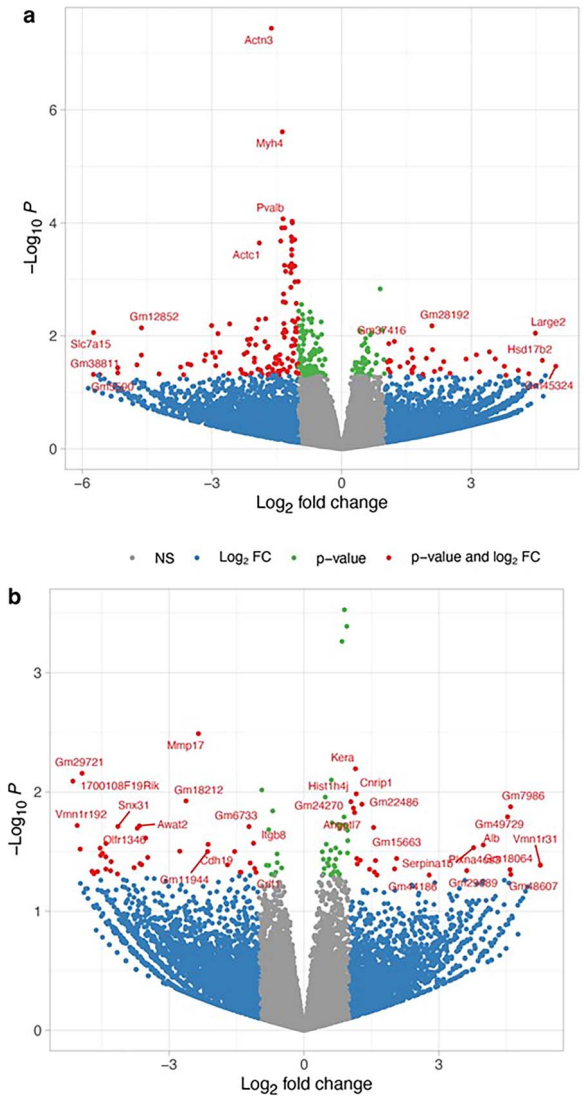


Figure 2. Genes differentially expressed in calvaria bones of miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ mice. (A) Male mice. (B) Female mice. Positive Log_2 fold change indicates higher expression level in miR21$^{\Delta Ot}$ mice. *P*-value of .05 and absolute log_2 fold change of 1 are used as cutoff in the volcano plot. Two of the genes, Actn3 (α-actinin-3) and Myh4 (myosin-4), had a false discovery rate less than 0.1 in male mice.

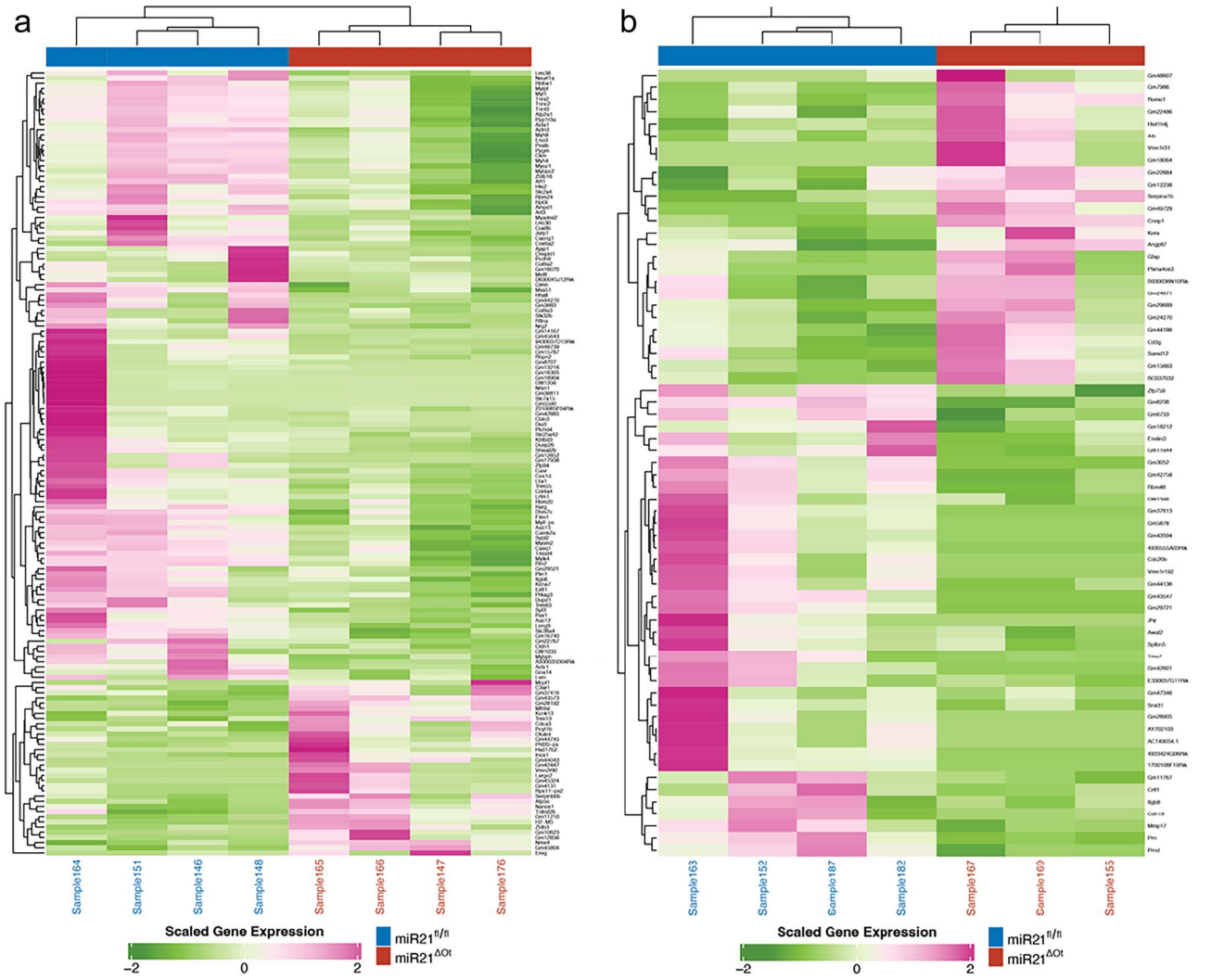


Figure 3. Heatmap of significantly differentially expressed genes between miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ mice. (A) Male mice. (B) Female mice. Significantly differentially expressed genes (DEGs) were defined as genes with *P*-value less than .05 and absolute $\log_2$ fold change larger than 1. About 152 DEGs were detected in male mice, while 65 DEGs were detected in female mice. For both male and female mice, the mice can be hierarchically clustered into 2 groups: miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ based on these DEGs.

In addition to the expected osteocyte-specific genes (Figure S3A), we detected the expression of genes purportedly expressed in other cell types (Figure S3B–H). However, it should be noted that most of these genes have been reported to be expressed by osteocytic cells and/or primary osteocytes,[16] raising the possibility that they originate from the bone cells themselves, and not from contaminated cells. Our study also showed that the osteoblastic gene Kera (encoding for keratocan) and the skeletal muscle Myh7 (encoding for myosin heavy chain β) only increased in miR21-deficient females. On the other hand, the expression of the genes enriched in muscle Actc1 (encoding for actin alpha cardiac muscle 1) and Actn3 (encoding for alpha actinin 3) was significantly lower only in male miR21-deficient animals compared to littermate controls, whereas the difference in Tcap (encoding for Titin-Cap) did not reach significance ($P = .055$). Altogether, this evidence gives further support to the sex-dependent effects of miR21 deletion. However, we cannot determine whether these changes are a direct consequence of the absence of miR21, are dependent on other genes directly affected by miR21, or are due to changes in the population of cells that express different levels of the genes.

For females, only 65 genes had *P*-value less than .05 and absolute log2 fold change larger than 1 (Figure 2B). The remaining 7 female samples can be clustered into miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ groups by the 64 genes (Figure 3B, Table S2). However, we did not see any significant KEGG or GO pathways including 5 or more significant genes.

After combining male and female data together and adjusting the gender difference, we found 150 significant genes (Table S3). However, these samples cannot be hierarchically clustered into miR21$^{fl/fl}$ and miR21$^{\Delta Ot}$ groups with the 150 genes. No KEGG or GO pathways were significantly enriched with significant genes.

Although there are some overlap significant genes between male and combined data and between female and combined (All) data, we did not find any overlap of significant genes between male and female data (Figure S4), which indicates that the effect of miR21 was sex specific. Thus, few genes had similar changes by miR21 in both male and female mice.

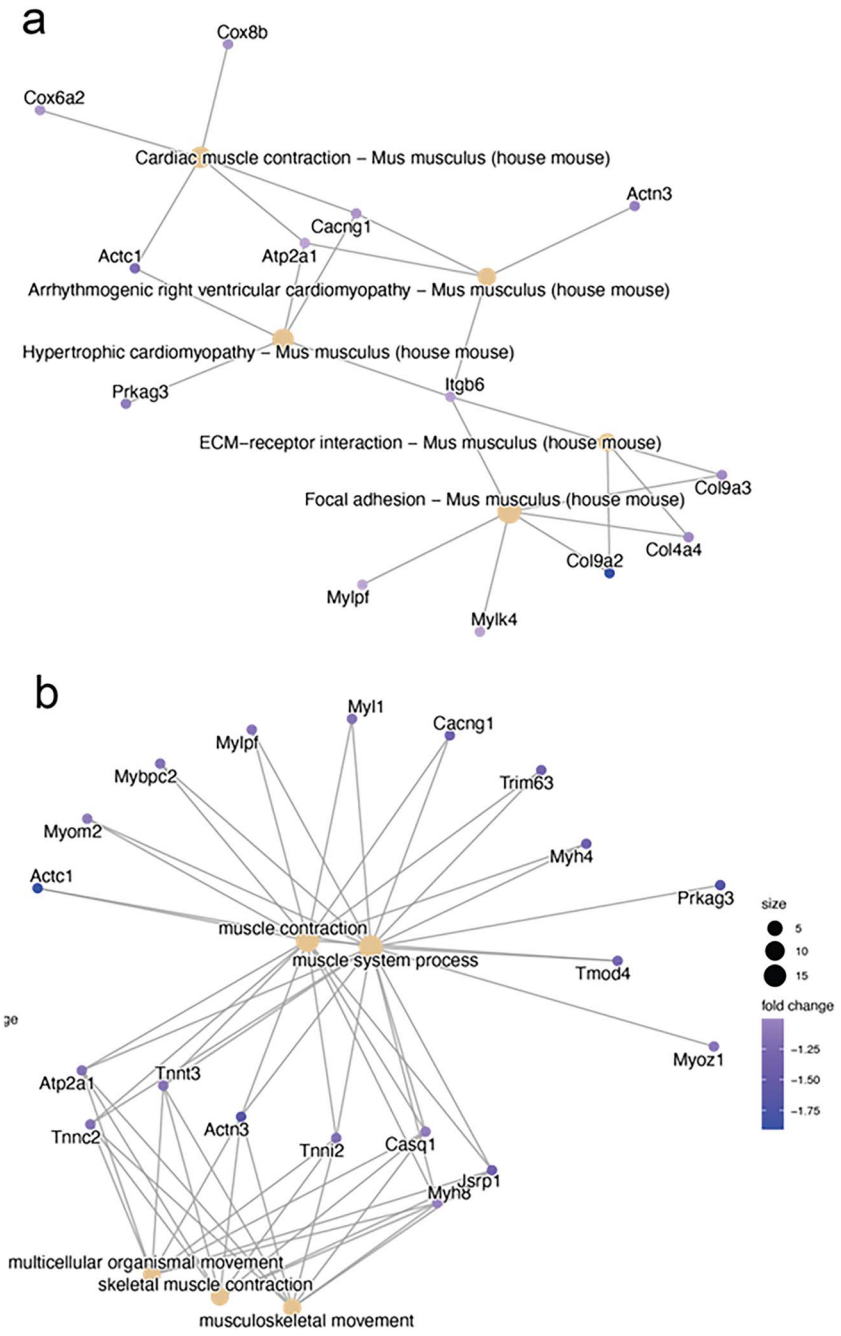


Figure 4. Net plot of DEGs in top significant pathways in male mice. (A) KEGG pathways (B) GO pathways. The dark yellow dots indicate the pathway, and the size of the dot is associated with the number of DEGs in the pathway. The small dots in the figure are DEGs. The $\log_2$ fold change of DEGs is illustrated in different shades. The $\log_2$ fold change of the DEGs in this figure are all negative, which indicates they have lower gene express in miR21$^{\Delta Ot}$ mice.

Discussion

miRs are widely expressed regulators of gene transcription, with known functions in the regulation of differentiation and activity of bone cells.[17] Among those miRs reported to influence bone cells, miR21 is the most abundant. Originally described as an oncogene, miR21 inhibits cell apoptosis, induces pro-survival autophagy, and is involved in metabolic reprograming, enhancing tumor progression.[18] In bone, miR21 has been shown to regulate both bone formation and bone resorption arms of bone remodeling.[19] We previously showed that deletion of miR21 from cells expressing an 8 kb fragment of the DMP1 promoter (mainly osteocytes) results in a sex-dependent phenotype, with reduced osteoblasts and osteoclasts in females (with no change in bone mass) and increased osteoblasts and osteoclasts in males (resulting in increased bone mass).[5] Further we showed that basal levels of miR21, as well as levels in miR21$^{\Delta Ot}$ mice, are lower in calvaria bones from males compared to female mice.[6] These differences are associated with divergent changes in gene expression in bones and levels of circulating chemokines. Long bone strength was higher in miR21-deficient male and female mice compared to the respective controls, although

the differences were higher for male mice. These results suggest that also at the bone structural/material level, the consequences of miR21 deletion are sex-dimorphic.

In order to assess the basis of the differential effect of miR21 deficiency at the transcriptomics level, we performed RNAseq analyses of calvaria bone preparations. We chose to use these bones because we found that miR21 levels were similarly decreased in male and female miR21$^{\Delta Ot}$ mice, whereas gene expression levels differentially changed in calvaria bones depending on the sex of the animals.[5] miRs are considered primarily translational repressors, but they can also induce mRNA degradation, thereby leading to the reduced expression of target genes.[20] It should then be expected that deletion of miR21 would lead to increased expression of target genes. However, we found that the expression of some genes in miR21$^{\Delta Ot}$ mice was higher than that in miR21$^{fl/fl}$ controls. Future studies would be needed to determine whether miR21 directly affects the expression of those genes, or whether the differences in expression are due to changes in the cell population in the calvaria bone in the presence and absence of miR21 expression. Further, the potential role of sex steroids on the modulation of gene expression in males vs females remains to be investigated.

Among the genes found to be differentially expressed with and without miR21 deletion, Actn3 and Myh4 were the only ones with a false discovery rate lower than 0.1. These 2 genes are associated with skeletal muscle function.[21,22] Both Actn3 and Myh4 have been shown to be expressed in high levels in skeletal muscle cells, a tissue not to be expected to be present in calvaria bone preparations. Yet, our pathway analyses showed that the genes differentially regulated in male mice samples were mostly associated with muscle function, suggesting that they might also be expressed in bone cells and, in particular, in osteocytes. Consistent with this, expression of Actn3 and Myh4 in bone preparations has been reported by Peter Croucher's group.[16] Similarly, a study by Ivo Kalajzic's group showed expression of numerous genes related to muscle function, development, and differentiation in calvaria bones.[23] Yet, the function of these so-called skeletal muscle genes on bone cells remains to be determined.

Our study shows that between 66 and 153 genes are expressed at significantly different levels between miR21fl/fl and miR21$^{\Delta Ot}$ mice (Tables S1–S3). Since miR-21 is often detected in proliferating cells and has been described as an oncogene,[24] it is possible that its function in terminally differentiated osteocytes is not that evident, and that a higher number of genes could be altered at earlier stages of the craniofacial development. Future studies will be needed to determine whether, indeed, deletion of miR-21 in proliferating osteoblast precursors, for example, has a more profound effect in the phenotype of the mice.

miR-21 share the same seed sequence as miR-590-5p and both promote osteoblastogenesis by targeting osteoblast precursors, raising the possibility that miR-590 might compensate for the absence of miR-21. However, no evidence has been reported on the action of miR-590 in mature osteoblasts and osteocytes, with the exception of regulation of Smad7 in human osteosarcoma MG63 cells.[25] On the other hand, we found a study in which miR-21 was deleted in cancer-derived cells, which lead to the concomitant changes in other miRs, including miR-4485-3p and miR-194-3p, without any changes in miR-590-3p or miR-590-5p levels, suggesting there is not compensation.[26] Future studies are needed to determine whether there is a cross-regulation between the 2 microRNAs in osteocytic cells.

In summary, our study suggests that, even though miR21 levels in calvaria bones are lower in male than in female control mice,[5] deletion of miR21 has a stronger effect on male transcriptome. These results are consistent with a positive effect on bone mass and remodeling, and a higher increase in bone strength in miR21$^{\Delta Ot}$ compared to miR21$^{fl/fl}$ male mice. On the other hand, although there are some genes significantly changing in females, they are not enriched in any pathways. Either there are no difference between the 2 groups in female, or the effect size is small, necessitating a larger sample size to detect genotype-dependent differences in the transcriptome.

Author contributions

Gang Peng (Data curation, Formal analysis, Methodology, Visualization, Writing—original draft, Writing—review & editing), Padmini Deosthale (Formal analysis, Investigation, Methodology, Writing—review & editing), Roquelina Pianeta (Formal analysis, Writing—review & editing), Hannah M. Messermith (Conceptualization, Formal analysis, Investigation, Methodology, Writing—review & editing), and Lilian I. Plotkin (Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing—original draft, Writing—review & editing)

Supplementary material

Supplementary material is available at *JBMR Plus* online.

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Conflicts of interest

None declared.

Data availability

All data are provided in the manuscript and the Supplementary Information.

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