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The miR221/222 family regulates vascular smooth muscle cell calcification.

Introduction:

The process of vascular calcification shares many similarities with that of skeletal mineralisation, and involves the phenotypic trans-differentiation of vascular smooth muscle cells (VSMCs) to osteoblastic and chondrocytic cells within a calcified environment. Various microRNAs (miRs) are known to regulate cell differentiation, however their role in mediating VSMC calcification has yet to be fully understood.

Methods:

Murine VSMCs were cultured in calcifying medium containing 3mM Na2HPO4/NaH2PO4 for up to 14d. Calcium deposition was confirmed by alizarin red staining and quantitative HCL leaching.. miR-microarray analysis was undertaken to identify novel miRs modulated during VSMC calcification. RT-qPCR analysis confirmed changes in miR expression. Finally, VSMCs were transfected with mimics of miR221 (50nM) and miR222 (50nM), individually and in combination.

Results:

1. VSMC calcification in vitro is associated with increased expression of osteogenic markers

High Pi (3mM) induced a significant increase in VSMC calcium deposition at day 7 and day 14, as revealed by alizarin red staining (Fig.1A) and HCL leaching (Fig.1B). A significant increase in mRNA expression of *Runx2* (Fig.1C) , *Bmp2* (Fig.1D) and *PiT-1* (Fig.1E) was seen by 14 d. The up-regulation of these osteogenic markers in VSMCs cultured in calcifying medium confirms the validity of this in vitro model to study AMC. *Bmp9* mRNA expression was also significantly increased at 14 d in VSMCs cultured in calcifying medium (Fig.1F) .

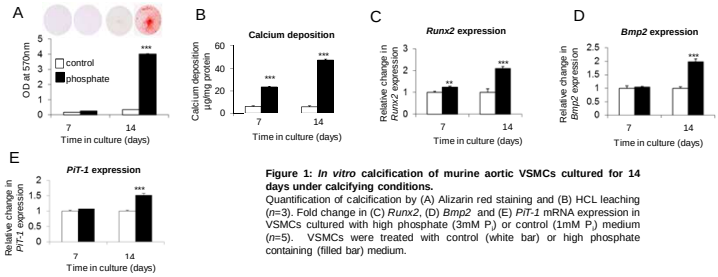

Figure 1: *In vitro* calcification of murine aortic VSMCs cultured for 14 days under calcifying conditions.
Quantification of calcification by (A) Alizarin red staining and (B) HCL leaching (n=3). Fold change in (C) *Runx2*, (D) *Bmp2* and (E) *PiT-1* mRNA expression in VSMCs cultured with high phosphate (3mM P_i) or control (1mM P_i) medium (n=5). VSMCs were treated with control (white bar) or high phosphate containing (filled bar) medium.

2. miR-microarray analysis

miR-microarray analysis revealed the significant down-regulation of a wide range of miRs by 9d of culture, including miR-199b (270 fold), miR-29a (168 fold), miR-221 (108 fold), miR-222 (81 fold), miR-31 (40 fold) and miR-30d (11 fold) (Table 1).

miRNA	Fold change from day 0
miR-379	222.5
mir-199b	269.8
let-7i	238
miR-16	192.5
miR-27b	195.1
miR-29a	168.2
miR-221	108.6
miR-199a	102.4
miR-674	139.2
miR-151	81.2
miR-100	95.3
miR-222	80.9
miR-22	90.3
miR-652	83.2
miR-130a	77.8
miR-382	69.7
miR-361	69.4
miR-27a	48.2
miR-24-2	43.8
miR-31	40.6

Table 1: miRNAs downregulated in VSMCs cultured for 9 days under calcifying conditions.

3. Verification of miR-array by RT-qPCR

A selection of miRNAs determined to be down regulated based microarray analysis were selected for qPCR validation. In agreement with the results of the micro-array these data indicate significant down-regulation of miR221 (32.4%; p<0.01), miR222 (15.7%; p<0.05), miR31 (43.7%; p<0.01), miR-27a (30%; p<0.05) and miR-24-2 (23.7%; p<0.05) expression was detected after 14 days culture in high phosphate medium (Figure 1). Interestingly, whereas in the microarray study miR199b was shown to be the most significantly down regulated, when tested by qPCR, no change in miR-199b expression was detected.

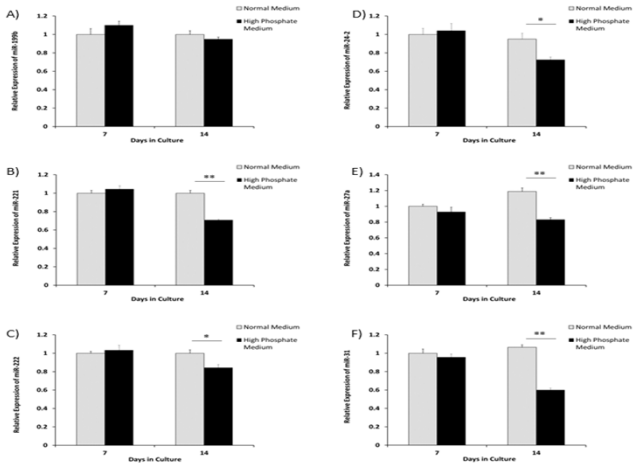

Figure 1: RT-qPCR verification of miR-array. Fold change in expression of (A) miR-199b, (B) miR-221, (C) miR-222, (D) miR-24-2, (E) miR-27a and (F) miR-31 in VSMCs cultured in control or high phosphate medium.

4. The miR221/222 family regulates VSMC calcification

To determine if miR221 and miR222 have a role during calcification of VSMCs *in vitro*, we transfected VSMCs with mimics of miR221 (50nM) and miR222 (50nM), individually and in combination, alongside a miR-ve control transfection and incubated in high phosphate medium for 7 days. All cells showed significant calcium deposition after 7 days treatment (miR221 p<0.05; miR222 p<0.001; miR221/222 p<0.01; miR-ve p<0.01). An increase in calcium deposition was observed when cells transfected with miR-ve were compared to cells co-transfected with miR221 and miR222 (2 fold; p<0.05). Interestingly, those cells transfected with individual miR221 and miR222 mimics did not show any significant difference when compared to the miR-ve treated cells (Fig. 2A).

5. Increase in calcification is independent of Runx2 and Msx2

Cells treated with miR221 and miR222, in combination and individually, were harvested at Day 0 and Day 7 of high phosphate (3mM NaPi) treatment for RNA analysis and compared to cells transfected with miR-ve. The present study shows a significant increase in Runx2 (Fig 2B) and Msx2 (Fig 2C) mRNA expression in all cells following 3 days in high phosphate medium but no difference in gene expression when the different miR treatments are considered.

6. Changes in expression of calcification regulators consistent with phenotype

Increased expression of *Alp* was observed within 24hrs of transfection (p<0.001) when compared to cells transfected with miR-ve (Fig 2D). Only by Day 3, was *Alp* expression significantly increased in miR221 (p<0.001) and miR222 (p<0.001) when compared to miR-ve treated cells. Conversely, in cells treated with individual miR221 (p<0.001) and miR222 (p<0.05) showed a significant increase in Enpp1 expression at Day 0 whereas no change was seen in cells transfected with miR221/222 in combination. Expression levels of P,T-1 were significantly increased by Day 7 in cells treated with miR-ve (p<0.001) when compared to cells treated with all combinations of miR221 and miR222.

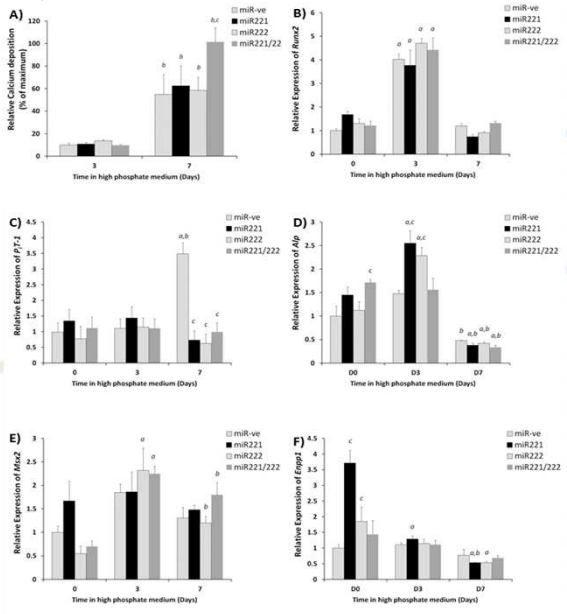

Figure 2: The miR221/222 family regulates VSMC calcification.

(A) Calcium deposition and Fold change in expression of (B) Runx2, (C) Pit-1, (D) ALP, (E) Msx2 and (F) Enpp1 in VSMCs transfected with mimics of miR221 and miR222, and cultured in high phosphate medium.

Conclusions:

miR-221/222 down-regulation may induce the phenotypic transition of VSMCs to osteoblastic and chondrocytic cells during calcification.

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