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Satb1 promotes osteoclastogenesis by recruiting CBP to upregulate miR-223 expression in chronic kidney disease-mineral and bone disorder

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Chronic kidney disease-mineral and bone disorder (CKD-MBD) is a complex disease which is associated with alterations of bone and mineral metabolism. miR-223 is implicated in both vascular calcification and osteoporosis. In the present study, we investigated the influence of Stab1 gene on miRNA-223 expression in osteoclastogenesis. Differentiation of monocyte/macrophage precursors was assessed by using RAW264.7 cells and peripheral blood mononuclear cells (PBMC). TRAP activity and bone resorption were used to measure osteoclast activity. Overexpression of Satb1 induced a marked increase in osteoclastogenesis in RAW cells ($P<0.01$) and a decrease in miR-223 expression ($P<0.01$). In contrast, upregulation of miR-223 increased osteoclastogenesis, as measured by osteoclast number ($P<0.01$) and TRAP activity ($P<0.001$). We showed that miR-223 affected the expression of its target genes NFIA and RhoB ($P<0.01$). Silencing of Satb1 promoted the expression of the osteoclast marker gene OPG and inhibited the expression of NF-κB ($P<0.01$) and TNF-α ($P<0.001$). These results were confirmed by measuring bone resorption activity of human PBMC differentiated into osteoclasts where Satb1 suppression inhibited the differentiation of PBMC cells. We have shown that Stab1 modulates osteoclatogenensis by regulating the expression of miR-223. Thus, upregulation of this miRNA to selectively increase osteoclast-like activity in calcified vessels of CKD-MBD could alleviate vascular calcification without altering bone structure.

1. Introduction

Chronic kidney disease–mineral and bone disorder (CKD-MBD) is a systemic disorder of mineral and bone metabolism encompassing one or more of the following conditions: aberrations in calcium, phosphorus, parathyroid hormone (PTH), or vitamin D metabolism; abnormalities in bone mineralization, volume linear growth, strength, and turnover; and extra-skeletal calcification (Levey et al. 2003; Moe et al. 2006). Accumulation of phosphorus, PTH, and FGF23, leads to formation of vascular calcification, endothelium dysfunction and bone structure changes which in turn result in cardiovascular complications (Miller 2014). Therefore, CKD-MBD is associated with increased mortality and fracture risk (Miller 2014). CKD-MBD not only increases the incidence of cardiovascular diseases, but also decreases the quality of life (Massy et al. 2005). Vascular calcification (VC) is identified as a major contributor to cardiovascular disease in patients with end stage renal disease (ESRD). The pathophysiology connected to bone turnover imbalance in CKD patients affecting vascular smooth muscle cells (VSMC) result into calcifying cells (Giachelli 2009). Apart from the disturbances in mineral metabolism (including hyperphosphatemia, metabolic acidosis, etc), inflammatory processes are also common during CKD which include rise in parameters like C-reactive protein (CRP) (Jalal et al. 2012), interleukin 6 (IL-6) (Lee et al. 2015), and interleukin 8 (IL-8) (Kiu Weber et al. 2014). Uremic toxins have been shown to play an important role in the development of CKD-MBD (London 2012). However, the pathogenesis is still not clear and it is necessary to understand the underlying mechanism for the clinical evaluation and treatment of CKD-MBD.

microRNAs (miRNAs) are small RNAs that generally have a post-transcriptional inhibitory effect on gene expression and are known as key regulators of numerous physiological and pathological mechanisms (Bartel 2009), including cardiac and vascular smooth muscle cell (VSMC) physiology (van Rooij 2011). Some studies propose that miRNAs are involved in the development of vascular damage in CKD (Goettsch et al. 2013). Some of them like miR-143, miR-145 and miR-223 are known to be key regulators of VSMC growth and proliferation of endothelial cell plasticity and damage (Rangrez et al. 2011; Taibi et al. 2014). It is seen in some animal experiments that there is an association between the decrease of miR-125b, -145 and -155 and vascular calcification (Chen et al. 2013). The importance of miRNAs in CKD-MBD is supported by their role as key regulators in function and differentiation of osteoblasts and osteoclasts during bone development and homeostasis (Hackl et al. 2016; Kapinas and Delany 2011).

In *in vitro* models of VC, it has been reported that the expression of miR-223 was upregulated when the smooth muscle cells were exposed to the uremic toxin inorganic phosphate (M'Baya-Moutoula et al. 2015; Rangrez et al. 2012). Similarly, in *in vivo* studies in a CKD mice model it was seen that miR-223 expression was increased in the aorta (Taibi et al. 2014). It was also observed that uremic toxin inorganic phosphate, a major risk factor in CKD, reduced the differentiation of osteoclasts by decreasing miR-143 and miR-145 expression *in vivo* and *in vitro* (Rangrez et al. 2012). In this study it was seen that in terms of osteoclastogenesis, Pi could play a role in bone remodeling in CKD patients because Pi induced a marked decrease of osteoclastogenesis in RAW cells, with a concomitant decrease of miR-223 levels (Rangrez et al. 2012). In addition, it has been reported in the same study that miR-223 high expression promotes osteoclast formation (Rangrez et al. 2012). Thus, the high level of miR-223 may be a new therapeutic strategy for the activation of vascular wall in osteoclasts, reversing vascular calcification in CKD-MBD disease or other abnormal symptoms of bone. However, the regulation mechanism of miR-223 is not clear at present, and the functional role of Satb1 in CKD-MBD is not explored. In this study, we therefore decided to assess the role of Satb1 in the regulation of miR-223 in RAW264.7 cells.

2. Investigations and results

2.1. *OE Satb1 promotes osteoclast survival*

To determine the expression of Satb1, cells were divided into two groups: negative control (C–) which were cultured without RANKL, and positive control (C+) which were treated with recombinant murine RANKL. As shown in Fig. 1A, the expression of Satb1 was increased by approximately six times in C+ cells with the differentiation of osteoclasts by Day 5. As expected, an overexpression of Satb1 increase of approximately 10-fold in differentiated cells was observed in Satb1 cells only (Fig. 1B). But in the presence of anti-miR-223, a significant decrease (P<0.01) in the differentiation of RAW 264.7 cells was detected (Fig. 1C).

Since, TRAP is widely considered to be a marker of osteoclast biochemical activity and released in large quantities by osteoclasts. We next determined the impact on differentiation by transfecting RAW 264.7 cells with pre-miR-223 (to induce overexpression) and treated with either 3 mM PI or 50 ng/ml RANKL, or with RANKL plus Pi for 5 days, and then processed for TRAP staining. We also determined the impact of Satb1 expression on TRAP activity. It was seen that overexpression of Satb1 promoted osteoclast formation of TRAP-positive cells (Fig. 1D).

2.2. *siSatb1 inhibits osteoclast formation*

To determine the role of Satb1 in the regulation of osteoclast formation, we silenced Satb1 expression in RAW 264.7 by using the silenced vector. In Satb1-silenced cells, the expression of Satb1 was significantly decreased (P<0.01; Fig. 2A).

To test the hypothesis that high Satb1 inhibits osteoclastogenesis by modulating Satb1 expression, we treated RAW cells with anti-Satb1 for 2 h to knockdown the expression of Satb1, then treated cells for 48 h with 3mM Pi, or 1.1mM as control. Under these experimental conditions, siSatb1 inhibited differentiation of RAW 264.7 cells by half as compared to control (P<0.001; Fig. 2B). In siSatb1 cells, a significant and marked decrease (P<0.01) in TRAP activity by almost one half was seen. Thus, confirming siSatb1 is involved in osteoclastogenesis by detecting the activity of TRAP (P<0.01, Fig. 2C).

To determine the intracellular mechanism of action of Satb1, we investigated the effect of Satb1 modulation on mRNA levels of osteoclast marker genes like NF-κB, TNF-α and osteoprotegerin (OPG). Interestingly, OPG had inhibitory effects on osteoclastogenesis and, as expected, it is increased in siSatb 1 cells by a marked 4-fold increase in OPG levels was seen as compared to control and oeSatb1 cells. In oeSatb1 cells the expression of NF-κB and TNF-α was increased approximately by 2- and 4-times, respectively. siSatb1 promoted the expression of osteoclast marker genes osteoprotegerin (OPG) and inhibits the expression of NF-κB and TNF-α (Fig. 2D).

2.3. *Satb1 increases the expression of miR-223*

In oeSatb1 cells the relative expression of miR-223 was increased by four times as compared to siSatb1 and control (Fig. 3A). Thus, it shows oeSatb1 promoted the expression of miR-223. We next investigated the effect of miR-223 regulation on the reported target RhoB. Satb1 inhibited the expression of RhoB and NFIA which are target genes of miR-223 (Fig. 3B). Western blot analysis detected the expression of RhoB and NFIA which showed that Satb1 inhib-

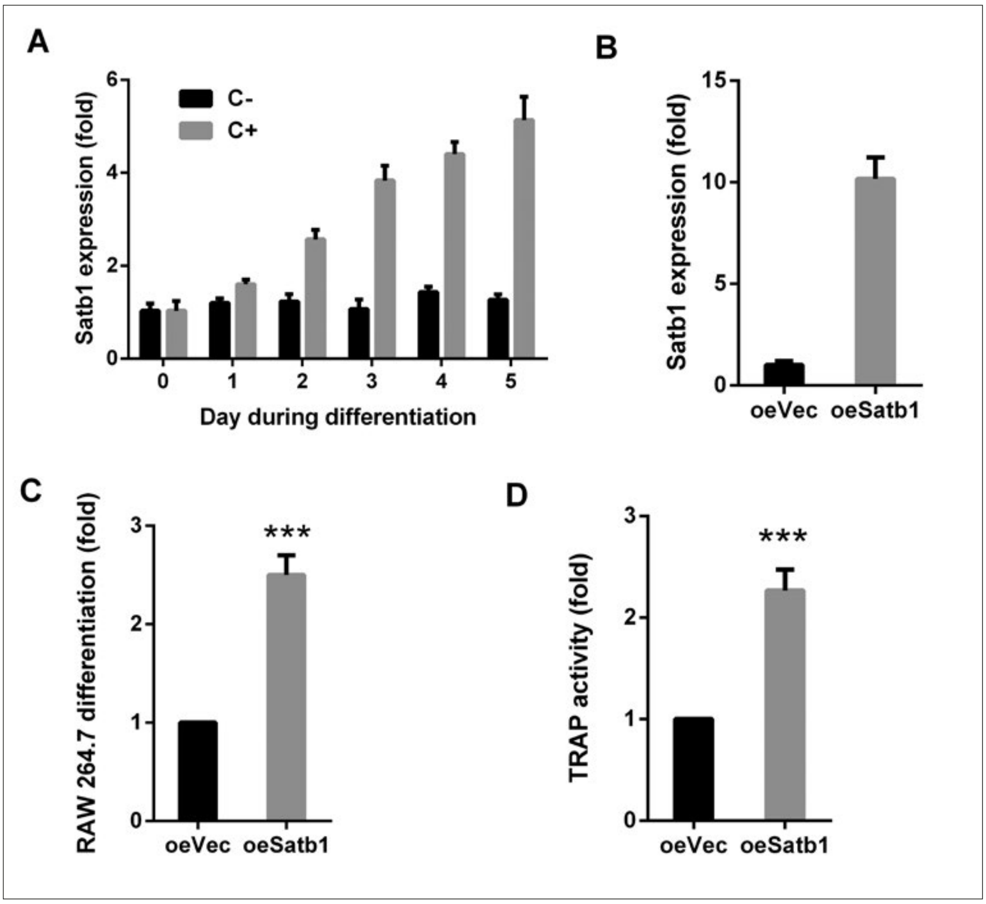

Fig. 1: Satb1 induces a marked decrease in osteoclastogenesis in RAW cells with a concomitant decrease in miR-223 levels. A. Satb1 promotes RANKL-induced differentiation of RAW264.7 cells. q-PCR analysis of osteoclastogenesis-related miRNAs in RAW264.7 cells cultured in DMEM containing 10% FBS for 5 days in the presence of soluble RANKL (50 ng/ml) and treated with 3mM of Pi compared to control cells not treated with RANKL (C-). B. Satb1 expression levels in RAW264.7 cells treated with Satb1. C. Effect of vec on differentiation of RAW264.7 cells. D. Impact of vec on TRAP activity. ***P <0.001.

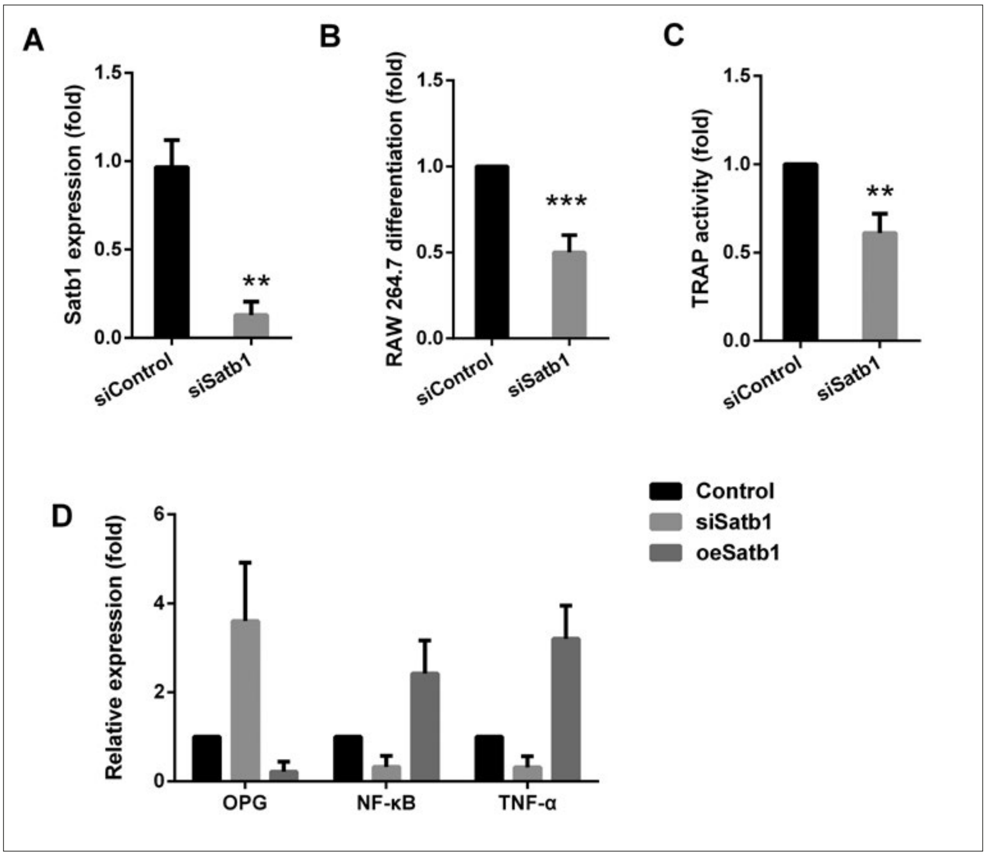

Fig. 2: Satb1 deletion causes inhibition of osteoclast formation. A. Satb1 knockdown downregulates the pluripotency-related genes and upregulates the differentiation-related genes. B. RAW264.7 cells were transfected with siSatb1 and differentiation rate was measured compared to control. C. RAW264.7 cells stained for TRAP expression. Quantification of multinucleated osteoclast differentiation from RAW264.7 cells using TRAP activity was assessed in each well. D. Relative expression of OPG, NF-κB and TNF-α levels were measured. **P <0.01, ***P <0.001.

ited the expression of RhoB and NFIA by promoting the expression of miR-223 (Fig. 3C). It was seen that in the cells treated with antiSatb1 were enriched 5 times more (P<0.05) compared to IgG cells (Fig. 3D). This shows that Satb1 deposits in the promoter region of miR-223.

2.4. *Satb1 recruits CBP and promotes gene transcription*

To identify the molecular mechanism of Satb1 in the regulation and differentiation in RAW cells, we did a Western blot analysis to identify the miR-223 interactors. Satb1 combined with CBP (CREB-binding protein) and the enrichment increased significantly (P<0.001) by four times as compared to IgG alone (Fig. 4A). Similarly, CBP combined with Satb1 and the enrichment increased significantly (P<0.01) as compared to IgG alone (Fig. 4B). Chromatin immunoprecipitation (ChIP) analysis showed that CBP combines with Satb1. CBP deposits in the promoter region of miR-223 (Fig. 4C). These observations suggested that CBP maintains RAW cells pluripotency through suppression of the expression of developmental genes. siSatb1 inhibited the deposits of CBP in the promoter region of miR-223 (Fig. 4D). siCBP inhibited the transcriptional activation of H3K27Ac in the miR-223 promoter region (Fig. 4E). As shown in Fig. 4F-4H oeSatb1+siCBP and oeSatb1+si-miR-223 inhibited osteoclast formation, siSatb1+-si-miR-223 mimic promoted osteoclast formation.

2.5. *Inhibition of RANKL-induced osteoclastic bone resorption of PBMC differentiated osteoclasts by Satb1*

To study the effects of Satb1 on the ability of osteoclasts to resorb bone, PBMC were cultured for 14 days on bovine bone slices in the presence of RANKL (25 ng/ml), M-CSF (30 ng/ml), and were treated with 3 mM PI and transfected with SAtb1. Following culture, siSatb1 significantly inhibited (P<0.01) the number of TRAP-positive cells by PBMC differentiation (Fig. 5A). The effects of the Stab1 on osteoclastic bone resorption was assessed by measuring two parameters: number of bone resorption pits and resorption rate. siSatb1 significantly (P<0.01) inhibited osteoclastic bone resorption as measured by resorption pits counting (Fig. 5B). Similar results were obtained for the resorption rate (Fig. 5C). Precisely, this rate was significantly reduced (P<0.01) in the siSatb1 cells.

3. Discussion

CKD-MBD is majorly characterized by laboratory abnormalities, bone abnormalities and vascular calcification and it accounts for about 40% deaths (Omata et al. 2015). Several studies show that miRNAs have potential as diagnostic and therapeutic targets because they are involved in CKD-MBD (van Rooij and Olson 2007). Based on this understanding, in this study we explored the role of Satb1 in the regulation of osteoclast formation. Osteoclastogenesis originates from mononuclear precursors and is responsible for regulating the bone resorption process (Krum et al. 2010). It is therefore essential to understand the underlying mechanism of osteoclast differentiation and to identify agents which specifically block its differentiation in osteoclast-related diseases (Qi et al. 2014). We found that the expression level of Satb1 was gradually increased in the process of osteoclast differentiation in RAW 264.7 cells. Knockdown of Satb1 inhibited the formation of osteoclasts, whereas high levels of Satb1 promoted the formation of osteoclasts.

Osteoclast differentiation is mainly modulated by two cytokines i.e. a tumor necrosis factor (TNF) family cytokine-receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), and macrophage-colony stimulating factor (M-CSF) (Bonewald 2011;

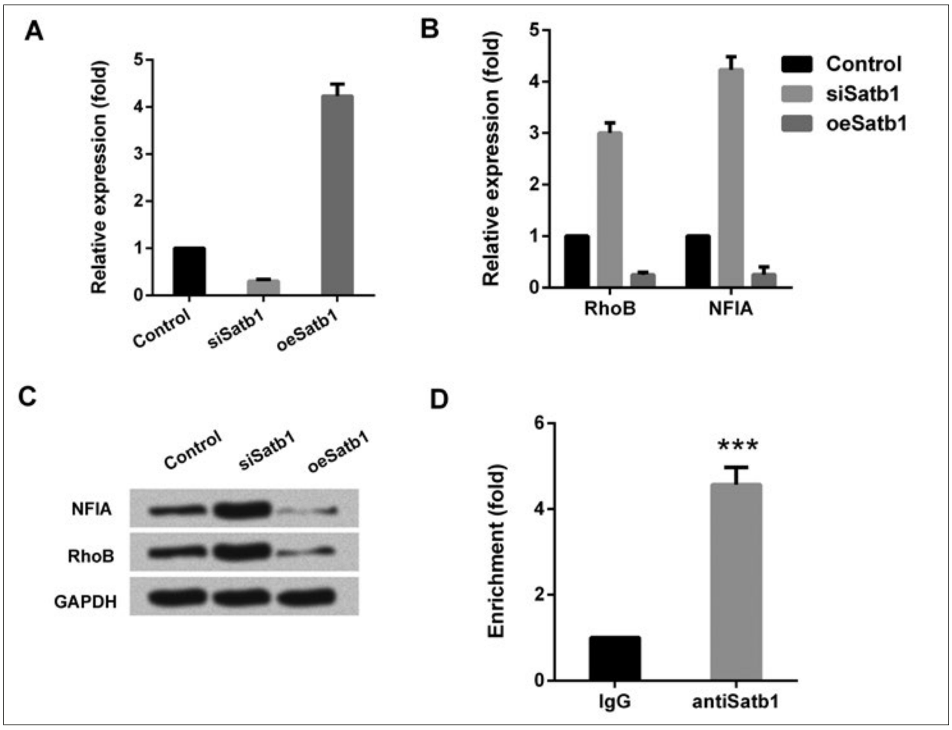

Fig. 3: miR-223 modulates the expression of osteoclast marker genes and reported targets of miR-223. A. After RNA extraction, RT-qPCR was performed to measure NFIA and RhoB using GAPDH as an internal reference. B. Total Relative expression of RhoB and NFIA. C. Western blot analysis to measure total proteins for RhoB and NFIA which were compared to GADPH. D. Enrichment fold in antiSAtb1 cells compared to IgG cells. ***P<0.001.

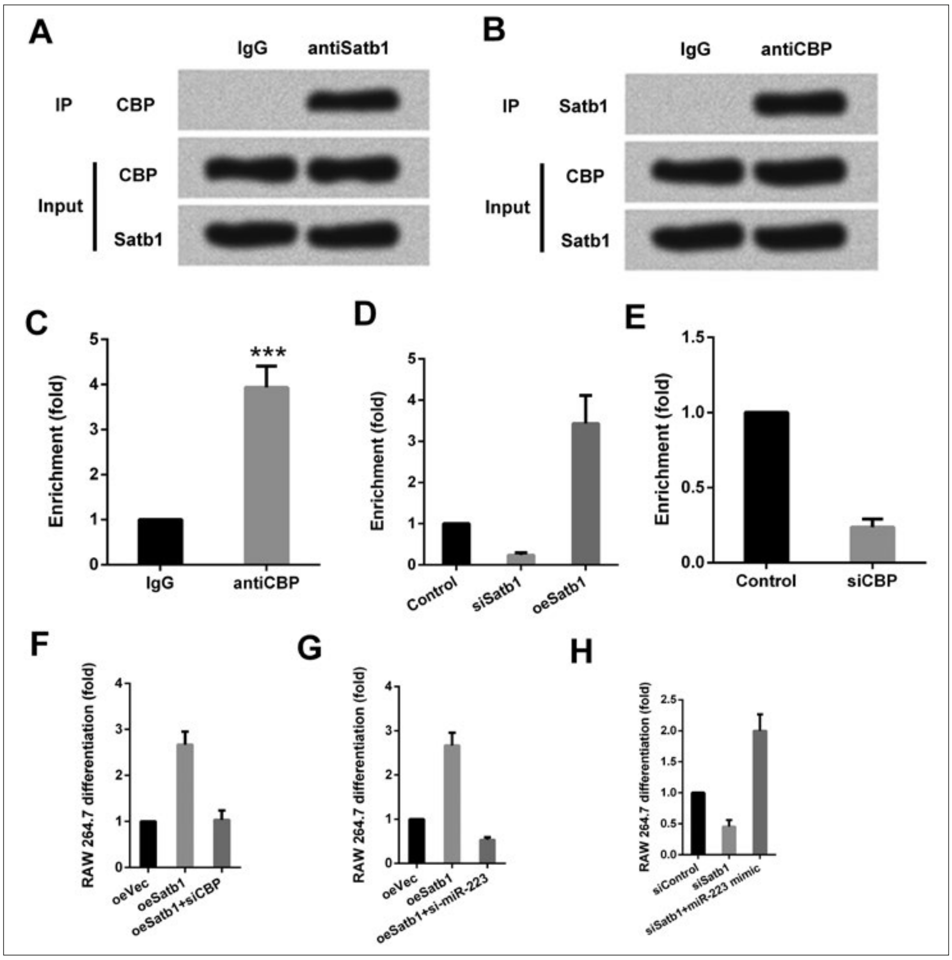

Fig. 4: Satb1 recruits CBP and promotes gene transcription. A. Satb1 combined with CBP as compared to IgG alone by using western blot analysis. B. CBP combined with Satb1 as compared to IgG alone by using western blot analysis. C. Enrichment fold in antiCBP cells compared to IgG cells. D. Enrichment fold in siSatb1 and oeSatb1 cells compared to control. E. Enrichment fold in siCBP cells compared to control. F. RAW264.7 cells were transfected with oeSatb1 and siCBP differentiation rate were measured compared to control. G. RAW264.7 cells were transfected with oeSatb1 and si-miR-223 differentiation rate were measured compared to control. H. RAW264.7 cells were transfected with siSatb1 and miR-223 mimic differentiation rate were measured compared to control. CPB: CREB-binding protein. *** P<0.001.

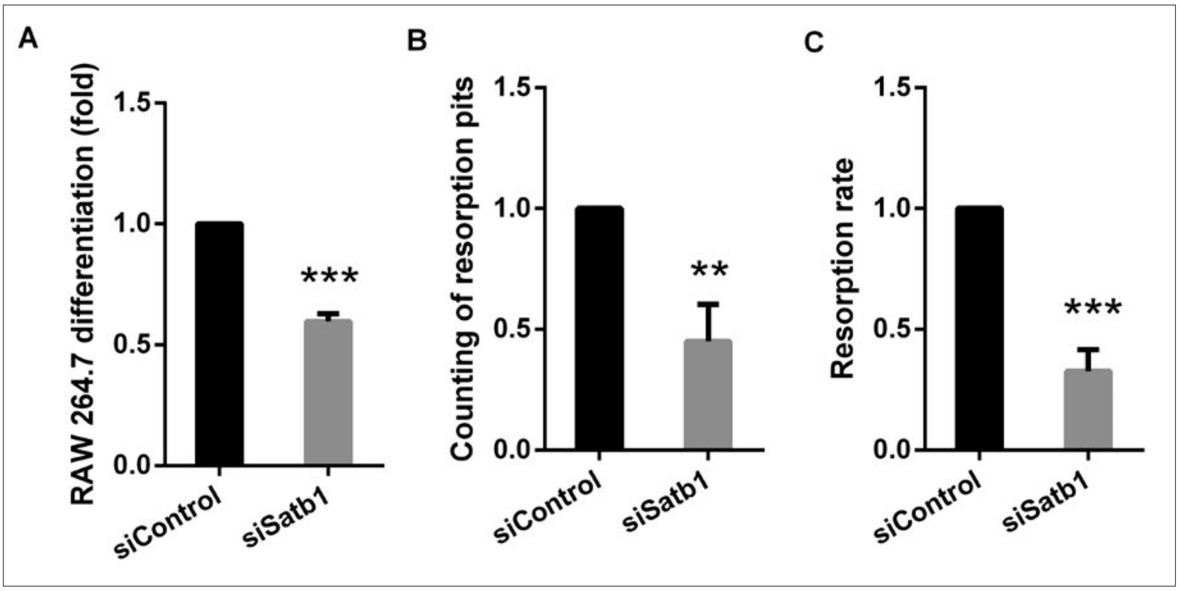

Fig. 5: Inhibition of RANKL-induced osteoclastic bone resorption of PBMC differentiated osteoclasts by Satb1. A. RAW264.7 cells were transfected with siSatb1 and differentiation rate was measured compared to control. B. Effect of Satb1 on resorption was measured by resorption pits counting. C. Effect of Satb1 on osteoclastic bone resorption, expressed in resorption rate (resorbed area/total area). **P<0.01, ***P<0.001.

Chambers 2000). RANKL, secreted from mesenchymal stem cell and osteoblasts, stimulates monocyte differentiation into osteoclasts (Chambers 2000). Following culture, siSatb1 significantly inhibited (P<0.01) the number of TRAP-positive cells by PBMC differentiation (Fig. 5a). The interaction of RANKL with its receptor RANK results in a cascade of intracellular events including nuclear factor kappa-light chain enhancer of activated B cells (NF-κB), mitogen-activated protein kinases (MAPKs), ionic calcium, and calcium/calmodulin-dependent kinase by recruiting the adaptor signal protein TNF receptor associated factor (TRAF6) (Hsu et al. 1999). As a result, a number of osteoclast-related marker genes, including tartrate resistant acid phosphatase (Trap), calcitonin receptor (Ctr), cathepsin K (Ctsk), and nuclear factor of activated T cells (Nfatc1), are upregulated (Hsu et al. 1999). In this study we investigated the effects of miR-223 modulation and Pi regulation on mRNA levels of three major players in osteoclastogenesis, NF-κB (Abu-Amer 2013), TNF-α (Kitaura et al. 2013) and OPG (a member of the TNF-receptor superfamily) (Montagnana et al. 2013). TNF-α, a potent bone-resorbing factor, is responsible for stimulating osteoclastic bone resorption both in *in vitro* and *in vivo* (Gronostajski 2000). It mediates RANKL stimulation of osteoclast differentiation by activating NF-kB through intracellular mechanisms overlapping those of RANKL (Zou et al. 2001). On the other hand, OPG is able to bind to RANKL and therefore competes for binding with RANK, thus blocking the inducing effect of RANKL on the osteoclast differentiation (Simonet et al. 1997). An increase in OPG could influence negatively osteoclastogenesis by reducing the differentiation of osteoclast precursors (Montagnana et al. 2013). Therefore, our findings are in line with these previous results that siSatb1 promoted the expression of osteoclast marker gene OPG and inhibited the expression of NF-κB and TNF-α. We also found that suppression of Satb1 could significantly inhibit the differentiation of PBMC cells into osteoclast cells and inhibit bone resorption. This suggests that, in contrast to TRAP activity, the most prominent features of osteoclast terminal differentiation, such as bone resorption, are not affected by an increase of miRNA-223.

In a study by Sugatani and Hruska (2007), it was seen that miR-223 is a key factor in osteoclastogenesis. It was also observed that reducing the total number of miRNAs decreased osteoclast number and increased trabecular bone mass (Sugatani and Hruska 2009). In another study it was seen that miR-223 is upregulated in rheumatoid arthritis synovium and its expression is affected during osteoclastogenesis (Shibuya et al. 2013). Our finding supports the previous results that overexpression of Satb1 and decreasing miR-223 expression inhibits osteoclastogenesis.

In a previous study it was found that NFIA (a negative regulator of M-CSF) is a target of miR-223 which facilitates osteoclast differentiation (Sugatani and Hruska 2007). It was also shown that NFIA is targeted by miR-223 in calcifying vascular smooth muscle cells (Rangrez et al. 2012). Here, in this RAW 264.7 cell study we have shown that Stab1 is able to modulate the expression of miR-223. We also observed that with miR-223 upregulation, osteoclastogenesis is increased as measured by osteoclast number and TRAP activity in RAW 264.7 cells.

Our study provides a new direction and theoretical basis for the treatment of CKD-MBD. Our findings suggest that Satb1 promotes miR-223 expression by recruiting CBP, promoting osteoclast formation and this could be a new perspective of exploring new strategies for CKD-MBD therapy.

4. Experimental

4.1. PBMC and RAW 264.7 cell culture and differentiation

As described previously, RAW 264.7 murine monocyte/macrophage cells (ATCC) were cultured (Mozar et al. 2008). In brief, cells were routinely cultured in DMEM containing 10% FCS. Media were supplemented with 1% glutamine/1% penicillin-streptomycin prior to use. For differentiation experiments, RAW 264.7 cells were gently scraped and seeded in 96-well plates (Corning) at a density of 5×10^3 cells per well in α-MEM differentiation medium supplemented with 10% FCS. The media was renewed after 2 h and few cells were treated and not with 50 ng/ml of recombinant murine RANKL. On the third day, media was replaced and cells were cultured for a total of 5 days. In this paper, cells cultured without RANKL are designated as the negative control (C–), and cells treated with recombinant murine RANKL which are differentiated are the positive control (C+). Differentiated cells treated with the scrambled RNA control are referred to as C+S. The absence of differentiation when RANKL was omitted was confirmed visually in all experiments.

Whole blood was provided by the Centre de Transfusion Sanguine of Amiens and human peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, density gradient centrifugation = 1.077). The PBMC culture was performed as previously described by Mozar et al. (2008). The cells were plated in 48-well plates at a density of 5×10^5 cells per well to a final volume of 300 mL of RPMI containing 10% FCS and were cultured for 14 days. Media was supplemented with 1% glutamine/1% penicillin-streptomycin prior to use. The media and added factors were replaced twice a week.

4.2. Tartrate-resistant acid phosphatase (TRAP) staining

As previously described, PBMC and RAW 264.7 cells were fixed and stained for TRAP, a marker enzyme of osteoclasts, using a commercially available kit (leukocyte acid phosphatase staining Kit 387 A, Sigma) (Mozar et al. 2008). Briefly, cells were washed with PBS and fixed in 3.7 % formaldehyde for 10 min. The cells were then incubated for 10–15 min (37 °C) in the staining solution and rinsed twice with

PBS. Multinucleated (>3 nuclei) TRAP-positive cells were counted as osteoclasts on microscopic examination.

4.3. *Determination of TRAP activity*

RAW 264.7 cells were lysed and incubated for 30 min in a reaction buffer containing paranitrophenyl phosphate (pNPP) (Mozar et al. 2008). The reaction was stopped with 0.3 N NaOH. ODs were then measured at 405 nm against a reference wavelength of 650 nm using a microplate reader (Biorad). The ODs were compared to a standard curve calibrated with paranitrophenol (pNP), and protein concentrations were quantified using the Biorad protein assay. Results were expressed as % of selected control (C+S).

4.4. *Bone resorption activity: pit area measurement*

PBMC were cultured as described above, and the resorption lacunae (pits) counting and percentage of resorbed bone area were estimated as previously described (Mozar et al. 2008). Briefly, cells were seeded on bovine bone slices (6 mm in diameter) pre-positioned in the wells. After 2 h, non-adherent cells were gently washed out from wells and 300 ml of fresh α-MEM supplemented with 10% FCS, human RANKL (25 ng/ml), and human M-CSF (30 ng/ml) were added, with or without Pi. Concomitantly, cells were transfected with scramble, pre-miR-223 or anti-miR-223. All treatments were given at the start of the experiment, and media and added factors were replaced twice a week. Cells were cultured for 14 days. At the end of the experiment, the number of osteoclasts (TRAP staining) and the resorbing activity were determined. For resorption analyses, TRAP positive osteoclasts were removed from the bone slices. The resorption lacunae (pits) were visualized by staining the bone slices with haematoxylin red and toluidine blue solution containing 1% sodium borate, and imaged by light microscopy. We counted as resorption pits an eroded surface of approximately the size of a multinucleated cell (with a number of nuclei ≥ 3), knowing that resorption pits could of course be larger. Total area of the investigated field, resorbed pit area, and the number of pits per field were determined manually with help of the software Bonoscan, developed and provided by Microvision which allowed us to spot the pits and the resorbed surface and the surface of the whole field. Results could be sorted out field by field or by meaning all the fields. The resorption rate was calculated by the software by dividing the resorbed surface by the whole surface.

4.5. *qRT-PCR*

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed by using the MultiscribeRTkit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C.

4.6. *Western Blot*

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Rabbit-anti-mouse CRT antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.7. *ChIP assay*

Quantitative ChIP was performed according to a standard protocol (Upstate). Sheared chromatin (sonicated to 200–500 bp) from 1×10^6 cells fixed in 1% formaldehyde was incubated with 5 µg antibody overnight at 4 °C followed by immunoprecipitation with salmon sperm DNA/protein agarose beads. After washing, elution and crosslink reversal, DNA from each ChIP sample and the corresponding input sample were purified and analyzed using qPCR.

4.8. *Immunoprecipitation assay*

Cells were transfected with the indicated plasmids and harvested. Cells were lyzed with ice-cold RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium desoxycholate, 0.1% SDS, 5 mM EDTA, 2 mM Phenylmethanesulfonyl fluoride (PMSF), 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml pepstatin A, 150 mM benzamidine, and 1% Nonidet P-40) for 30 min. Lysates were incubated with the indicated antibodies followed by immunoprecipitation with protein A/G agarose.

4.9. *Cell transfections*

The siPORT NeoFX transfection agent (Applied Biosystems) was used for miRNA knock-down and overexpression, according to the manufacturer's instructions and as previously described (Rangrez et al. 2012). RAW 264.7 cells were seeded in 24-well or 96-well culture plates (Corning) with 10^3 and 5×10^3 cells respectively. They were concomitantly treated with RANKL, as previously indicated, and/or transfected with irrelevant scrambled RNA, anti-miR-223 or miR-223 mimic. Cells were then collected and processed for further analysis after a total of 5 days in culture.

4.10. *Statistical analysis*

All experiments were repeated three times. The results of multiple experiments are presented as the mean±SD. Statistical analyses were performed using SPSS 19.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Conflicts of interest: None declared.

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