Int J Clin Exp Pathol 2017;10(7):7726-7733
www.ijcep.com /ISSN:1936-2625/IJCEP0054923

Original Article

Hydroxyapatite nanocrystals stimulate osteogenic differentiation in primary human aortic smooth muscle cells by activation of oxidative stress and the ERK pathway

Li Gong, Yaorong Liu, Jiaqi Qian, Zhaohui Ni, Wei Fang

Department of Nephrology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, People's Republic of China

Received April 8, 2017; Accepted May 20, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: Background: Hydroxyapatite nanocrystal (HN) deposition underlies the development of vascular calcification, which is an actively regulated process resembling bone formation. This study investigated the role of HNs in inducing osteogenic differentiation of primary human aortic smooth muscle cells (HASMCs). Methods: Primary HASMCs were incubated with HNs, cell osteogenic differentiation was evaluated by von kossa staining and calcium content. The expressions of SM-α-actin and bone markers, including runt-related transcription factor 2 (Runx2), osteopontin (OPN), osterix, and collagen 1 (COL1) were also determined. Antioxidants, ERK-specific inhibitor were used to examine whether oxidative stress and the ERK pathway were required for this transition. Results: Stimulation of HASMCs with HNs increased calcium deposition, expression of bone markers and decreased SM-α-actin expression. HNs produced reactive oxygen species (ROS) in HASMCs, as evaluated by fluorescent probe. Antioxidants inhibited HN-induced osteogenic differentiation. Furthermore, the inhibitor of the ERK pathway, PD98059, suppressed the effect of HNs on bone marker expression. Conclusions: These findings suggest that HNs stimulated osteogenic differentiation of vascular smooth muscle cells that build biomineralized deposits partly by activating oxidative stress and the ERK pathway.

Keywords: Hydroxyapatite nanocrystals, osteogenic differentiation, oxidative stress, the ERK pathway, primary human aortic smooth muscle cells

Introduction

Cardiovascular disease (CVD) leads to increased morbidity and mortality in patients with chronic kidney disease (CKD), and may in part due to excess calcification of the vessel wall [1, 2]. Vascular calcification occurs at two sites, the arterial intima and media. Calcification in the arterial media, which is primarily composed of vascular smooth muscle cells (VSMCs), is prevalent in CKD patients [3]. In recent years, vascular calcification has been considered to be an active process that resembles the formation of bone-like tissues in blood vessels [4]. VSMCs participant in the calcification process and undergo osteogenic differentiation as evidenced by upregulation of the expression of bone markers, such as runt-related transcription factor 2 (Runx2), bone morphogenetic protein-2 (BMP-2), osteopontin (OPN), osterix, and collagen 1 (COL1) [5].

Vascular calcification manifests as calcium phosphate deposits, which consist of hydroxyapatite in the primary mineralization phase, in arteries of patients with CKD [6]. It has recently been shown that hydroxyapatite nanocrystals (HNs) stimulate osteogenic differentiation in vitro [7]. Sage et al. [8], isolated deposits from a high phosphorus (P) medium, identified the deposits as hydroxyapatite varying in diameter from 30 to 500 nm, and showed that these nanocrystals caused an upregulation of BMP-2 and OPN. In our previous study [9], we also reported that HNs (isolated from uremic serum) could induce osteogenic differentiation of VSMCs. However, the mechanism by which these nanocrystals alter gene expression remains unclear.

Table 1. Specific primer sets

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	AAG GTG ACA GCA GTC GGT T	TGT GTG GAC TTG GGA GAG G
Runx2	CCA ACC AGC CTT ACC AAA C	TGC CTT TTC AGA GGA CCT AC
OPN	GCC GAG GTG ATA GTG TGG T	GAT GTC AGG TCT GCG AAA CTT
Osterix	GAT GGG GTA TCT CTT GAT TA	CAT AGG ACT TGA GGT TTC AC
COL1	GAC ATC CCA CCA ATC ACC TG	CGT CAT CGC ACA ACA CCT T

Increased oxidative stress and reactive oxygen species (ROS) play an important role in the pathogenesis of vascular calcification [10, 11]. Signaling of extracellular signal-regulated kinases (ERK) is part of ROS downstream [12]. Previously, the ERK pathway was reported to positively regulate bone development in vivo through Runx2 activation [13], besides, it also plays an essential role in differentiation of mesenchymal stem cells (MSCs) to osteogenic lineage [14-16]. In macrophages, HNs activate ERK to release tumor necrosis factor [17]. It is currently unknown whether HNs promote osteogenic differentiation of primary human aortic smooth muscle cells (HASMCs) through oxidative stress and the ERK pathway. We therefore investigated the role of oxidative stress and the ERK pathway in this transition.

Materials and methods

Reagents

Apocynin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Vitamins C and E and 2',7'-dichlorofluorescin diacetate were purchased from Sigma (St. Louis, MO, USA). Primary antibodies against SM-α-actin, Runx2, OPN, and osterix were purchased from Abcam (Cambridge, MA, USA), whereas primary antibodies against p-ERK, ERK, and PD98059 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Culture of primary HASMCs and treatment

Primary HASMCs were obtained from ScienCell (Carlsbad, CA, USA). Cells were cultured in basal media supplemented with 2% fetal bovine serum, smooth muscle cell growth supplement, and penicillin/streptomycin solution in 5% CO_2 at 37°C. Passage numbers 3 to 8 were used for experiments. To induce differentiation of HASMCs, HNs (Sigma, average size <200 nm) were added to media to reach final concentrations of 25, 50, or 100 μg/ml for 3 or 5 days. Cells cultured in the normal media were named as the control group.

Cell viability assay

A CCK-8 kit (Dojindo, Kumamoto, Japan) was used to detect cell proliferation and viability rates. Briefly, primary HASMCs were seeded in 96-well plates (5000 cells/well) and, after an initial 24 h, the normal media were switched to HN media at concentrations of 25, 50, or 100 μg/ml for 3 or 5 days. Next, a 10-μL volume of CCK-8 solution was added, and the mixture was incubated for 3 h. The optical density at 450 nm was also measured.

Calcium deposition in cells

Calcification was visualized using a von Kossa staining kit (Genmed, Plymouth, MN, USA). In addition, calcification was quantified by washing cells with phosphate-buffered saline (PBS), decalcifying the cells with 0.60 M HCl for 24 h, and then measuring the calcium content of HCl supernatants using a calcium colorimetric assay kit (Biovision, Milpitas, CA, USA). After decalcification, a BCA kit (Beyotime, Guangzhou, China) was used to measure the protein content, and the amount of calcium was normalized to total protein content.

Real-time polymerase chain reaction (PCR)

RNA of primary HASMCs was extracted by TRIZOL (Invitrogen, Carlsbad, CA, USA) and converted into cDNA using a reverse transcription kit (Promega, Madison, WI, USA). Primers for Runx2, OPN, osterix, and COL1 are shown in **Table 1**. PCRs were performed in the ABI ViiA7 system in a volume of 20 μL using the following parameters: 5 min of Taq activation at 95°F, followed by 40 cycles of PCR at 95°F, 15 s and 60°F 30 s. Relative mRNA quantity was calculated.

Immunofluorescence staining

Primary HASMCs were first washed in PBS and fixed with 4% formaldehyde. Subsequently, the cells were permeabilized with 0.2% Triton X-100 and blocked with 5% BSA. Next, the cells were incubated with primary antibodies (SM-α-actin, 1:100; Runx2, 1:100; OPN 1:200) at 4°C overnight followed by recognition with FITC (fluorescein isothiocyanate)-labeled secondary anti-

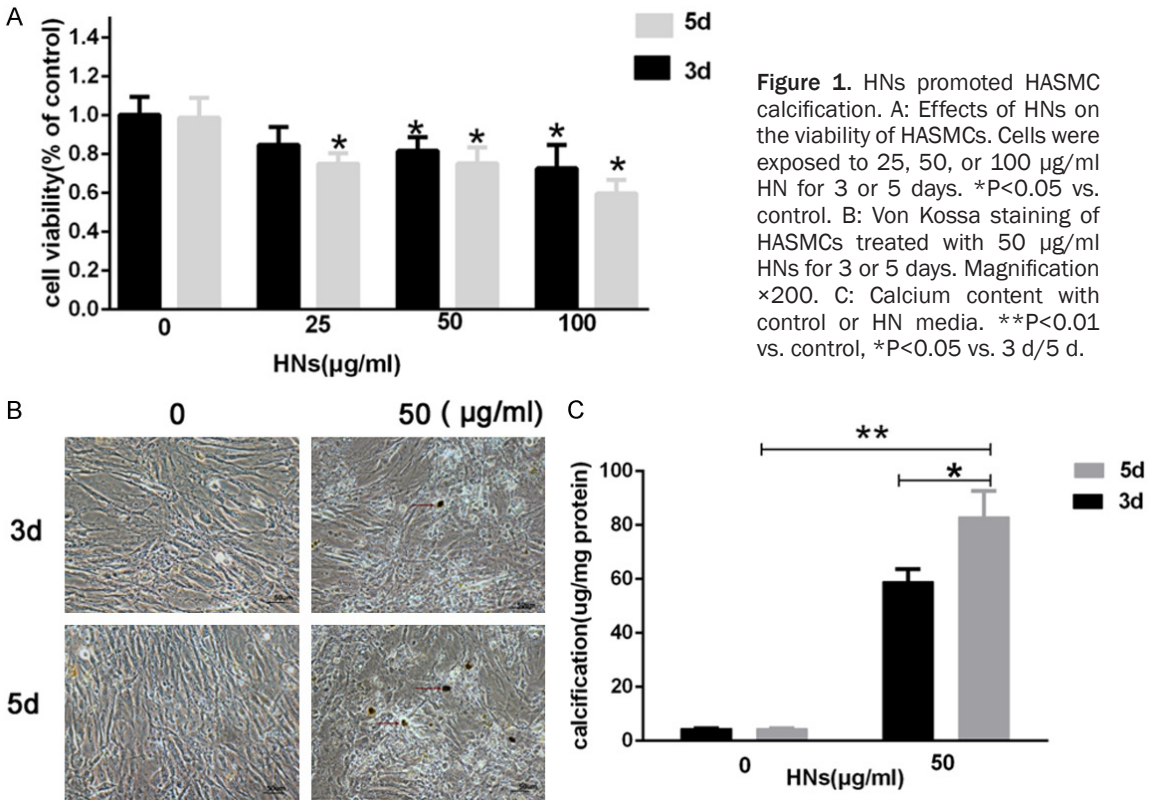

Figure 1. HNs promoted HASMC calcification. A: Effects of HNs on the viability of HASMCs. Cells were exposed to 25, 50, or 100 μg/ml HN for 3 or 5 days. *P<0.05 vs. control. B: Von Kossa staining of HASMCs treated with 50 μg/ml HNs for 3 or 5 days. Magnification ×200. C: Calcium content with control or HN media. **P<0.01 vs. control, *P<0.05 vs. 3 d/5 d.

bodies for 30 min in the dark. After incubation with 4',6-diamidino-2-phenylindole (DAPI), specimens were observed under a confocal scanning microscope (Leica TCS SP5). Fluorescence density was quantitatively analyzed using Image J software.

Western blot

Cell protein was extracted using lysis buffer supplement with protease and phosphatase inhibitor (Beyotime). The protein concentration was quantified by the BCA protein assay method. An amount of 20 μg of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4°C overnight followed by recognition with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Signals were visualized with an enhanced chemiluminescence (ECL) reagent (Cell Signaling Technology), and protein expressions were analyzed by blot densities using gelpro32 imaging.

Detection of ROS

Fluorescence intensity of dichlorofluorescein diacetate (DCF-DA), a ROS probe, was analyzed. Cells were incubated with either 50 μg/ml HNs for 3, 6, 12, 24, 36, and 48 h, or 50 μg/ml HNs for 24 h in the presence of antioxidants (e.g., 50 μmol/l apocynin or 200 μmol/l vitamin C plus 30 μmol/ml vitamin E) and then loaded using a 10-μmol/l probe at 37°C for 30 min. Fluorescence intensity was measured using ipwin 32 software. Antioxidant concentrations were chosen according to previous studies [18, 19].

ERK pathway in HN-induced osteogenic differentiation

Primary HASMCs were treated with 50 μg/ml HNs for 0, 15, 30, and 60 min, and then phospho-ERK1/2 was examined by western blot. We further investigated the role of the ERK pathway in HN-induced differentiation by pretreating HASMCs with 50 μmol/l PD98059, a potent ERK inhibitor, for 1 h and then incubating the cells with HNs for 5 days. Expressions of Runx2 and osterix were assessed following the incubation. To determine whether ROS participated in the mechanism of HN-induced osteogenic differentiation, we treated HASMCs with 50 μg/ml HNs in the presence or absence of antioxidants for 60 min and then determined phospho-ERK1/2.

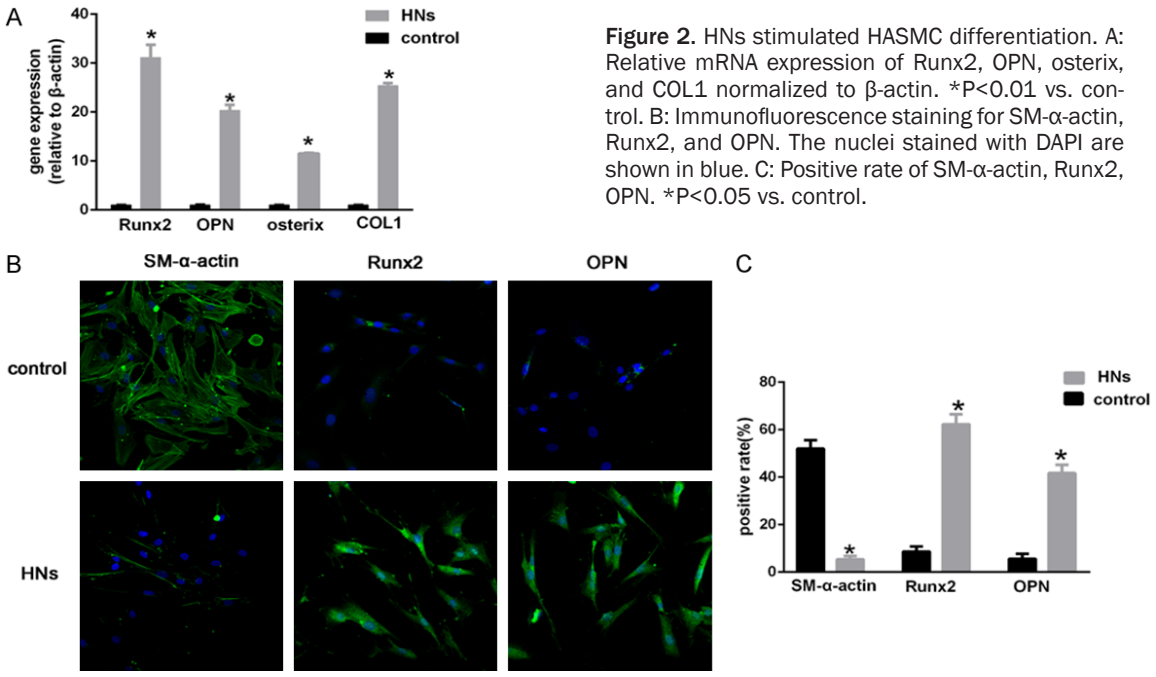

Figure 2. HNs stimulated HASMC differentiation. A: Relative mRNA expression of Runx2, OPN, osterix, and COL1 normalized to β-actin. *P<0.01 vs. control. B: Immunofluorescence staining for SM-α-actin, Runx2, and OPN. The nuclei stained with DAPI are shown in blue. C: Positive rate of SM-α-actin, Runx2, OPN. *P<0.05 vs. control.

Determination of COL1 in media by enzyme-linked immunosorbent assay (ELISA)

Cells were cultured with 50 μg/ml HNs in the presence or absence of 50 μmol/l PD98059 or antioxidants for 5 days. Cell culture supernatants were harvested for measuring protein concentration. All the processes were followed according to the protocol of the Human Type I Collagen Detection Kit (Chondrex, Redmond, WA, USA). The absorbance was measured at 450 nm on a microplate reader, and COL1 content was recorded as per protein in the supernatants (ng/ml).

Statistics

Data were from at least three experiments and expressed as mean ± SD. The t test was used to analyze difference between two groups. An ANOVA followed by a Student-Newman-Keuls test was also used to compare the groups. P<0.05 was considered significant with SAS software.

Results

HNs promoted primary HASMC calcification

The CCK-8 assay results indicate that the effects of HNs on the viability of HASMCs were time- and concentration-dependent. When cells were treated with 50 μg/ml HNs for 3 or 5 days, cell viability decreased to 81.4% and 76.1%, respectively, of that in the control group (**Figure 1A**). After being exposed to 50 μg/ml HNs for 3 or 5 days, HASMCs showed visible calcified nodules as indicated by von Kossa staining. Calcification was much more intense after 5 days compared with 3 days of HN treatment. There were no visible calcified nodules in the control group (**Figure 1B**). Calcium content assay results showed that calcium was present at 3 days (59.02 ± 5.13 μg/mg protein) and 5 days (83.34 ± 9.73 μg/mg protein) in HN-treated cells. Little calcium was detected in controls (**Figure 1C**).

HNs induced osteogenic differentiation in primary HASMCs

Real-time PCR results revealed that the mRNA expression of bone markers, including Runx2, OPN, osterix, and COL1, significantly increased after exposure to 50 μg/ml HNs for 5 days compared with control (**Figure 2A**). Protein expression was further detected using immunofluorescence (**Figure 2B**, **2C**). HASMCs in the normal media expressed the characteristic smooth muscle marker SM-α-actin, without expression of Runx2 or OPN. When cultured in HN media, cells developed higher levels of Runx2 and OPN and lower levels of SM-α-actin. All these results suggest that HASMCs experienced osteogenic differentiation when treated with HNs.

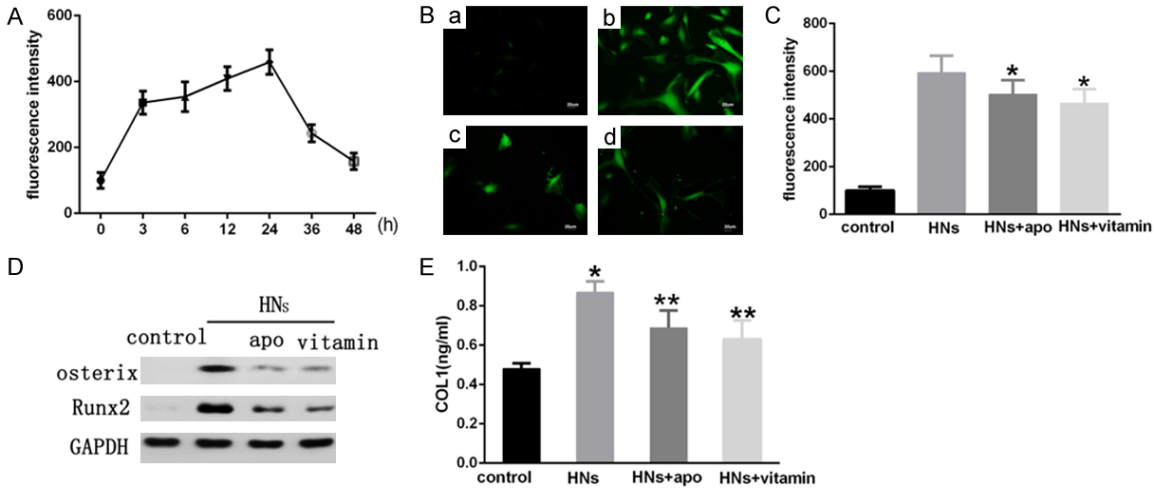

Figure 3. ROS regulated HN-induced transition. A: Time course of HN-induced ROS. B: ROS production detected by DCF-DA. Images (a-d) showing control, HNs, HNs + apocynin, and HNs + vitamin C plus vitamin E, respectively at 24 h (×200). C: Fluorescence intensity of aforementioned four groups. *P<0.05 vs. HN group. D: Effect of antioxidants on HN-induced expression of Runx2 and osterix determined by western blot. E: Changes of COL1 in cell culture supernatants after supplement with antioxidants. **P<0.05 vs. HN group.

ROS production in HN-induced osteogenic differentiation

To determine the effect of ROS in HN-induced osteogenic differentiation in HASMCs, we first observed that HNs increased ROS production over a period of 48 h (**Figure 3A**). Twenty-four hours of treatment with antioxidants markedly inhibited HN-induced ROS (**Figure 3B**, **3C**). Moreover, western blot analysis revealed that the expression of Runx2 and osterix induced by HNs was decreased by apocynin (0.436 ± 0.001 vs. 1.575 ± 0.005 and 0.109 ± 0.001 vs. 0.492 ± 0.003, respectively; *P<0.05) and vitamin C plus vitamin E (0.373 ± 0.003 vs. 1.575 ± 0.005 and 0.104 ± 0.004 vs. 0.492 ± 0.003, respectively; *P<0.05) (**Figure 3D**). In accord with the data of the western blot analysis, ELISA results indicated that apocynin or vitamin C plus vitamin E also decreased the expression of COL1 (0.688 ± 0.050 vs. 0.869 ± 0.032 ng/ml and 0.630 ± 0.055 vs. 0.869 ± 0.032 ng/ml, respectively; *P<0.05) induced by HNs (**Figure 3E**).

ERK pathway mediated HN-induced osteogenic differentiation

As shown in **Figure 4A**, HNs activated phospho-ERK1/2 in primary HASMCs. To further evaluate if ERK signaling mediated the HN-induced osteogenic differentiation, we tested the effect of ERK-specific inhibitor PD98059 on HN induction of bone marker expression. The results show that 50 μmol/L of PD98059 decreased the expression of Runx2 and osterix (0.367 ± 0.013 vs. 1.512 ± 0.056 and 0.114 ± 0.006 vs. 0.476 ± 0.022, respectively; *P<0.05) and COL1 (0.658 ± 0.050 vs. 0.869 ± 0.032 ng/ml, respectively; *P<0.05) induced by HNs (**Figure 4B**, **4C**). In addition, HASMCs showed phosphorylation of ERK1/2 at 60 min after 50 μg/ml HN stimulation. This phosphorylation was inhibited by antioxidants, suggesting ROS was required for ERK activation by HNs in HASMCs (**Figure 4D**, **4E**).

Discussion

The results of the present study show that HNs stimulated osteogenic differentiation of primary HASMCs as evaluated by increased calcium deposition and the expression of bone markers, including Runx2, osterix, OPN, and COL1. Furthermore, by culturing primary HASMCs in the presence of antioxidants or an ERK-specific inhibitor, we also demonstrated that HN induction of osteogenic gene expression was mediated partly by activation of oxidative stress and the ERK pathway.

Vascular calcification is commonly encountered in CKD patients, and is also a complicated process whose regulatory mechanisms have not been well understood. It has been suggested that HNs could promote calcification through

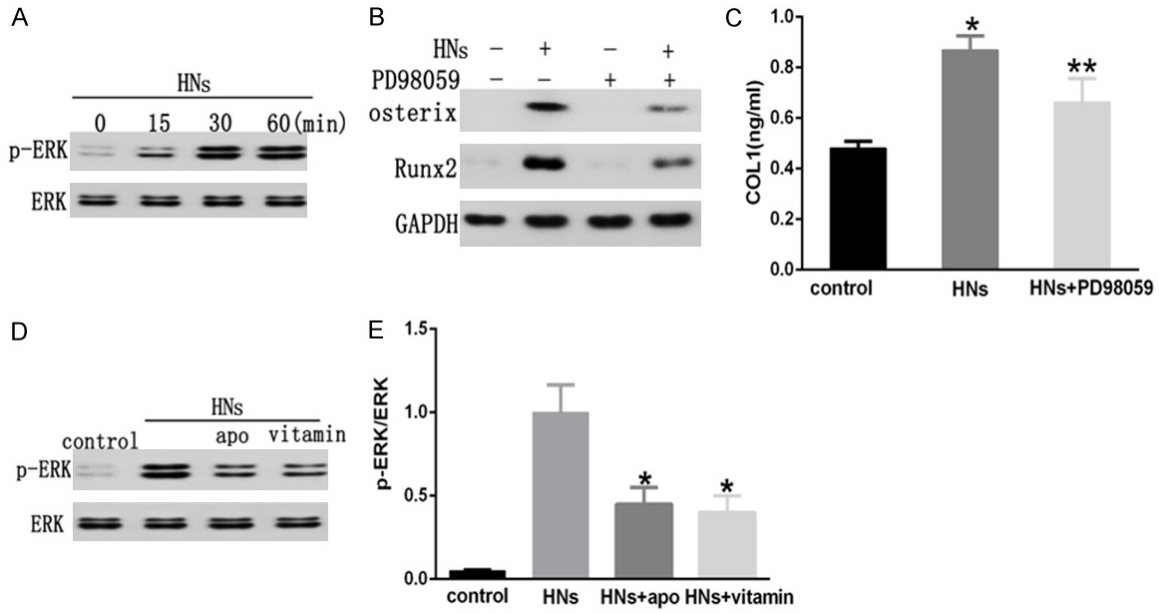

Figure 4. ERK pathway regulated HN-induced transition. A: HN-induced ERK1/2 phosphorylation in HASMCs. B: Effect of PD98059 on HN-induced expression of Runx2 and osterix determined by western blot. C: Changes of COL1 in cell culture supernatants after inhibition of ERK pathway. *P<0.05 vs. HN group. D: Effect of antioxidants on HN-induced ERK1/2 phosphorylation at 60 min. E: Antioxidants reduced p-ERK/ERK ratio induced by HNs. *P<0.05 vs. HN group.

several mechanisms, including increased inflammatory cytokine release of macrophages [17], inducing apoptosis in VSMCs [20], or stimulating osteogenic differentiation in VSMCs [7-9].

Similarly, we found that incubation of primary HASMCs with HNs decreased SM-α-actin expression and increased expression of bone gene/proteins, such as Runx2, osterix, COL1, and OPN. Runx2 is a key transcription factor for osteogenic differentiation, as it directly or indirectly modulates a number of other genes specific for osterix, COL1, as well as OPN [21]. Osterix is also an important transcription factor that acts downstream of Runx2 [22]. COL1 present in the extracellular matrix (ECM) provides compartments where minerals grow, whereas OPN inhibits calcification of VSMCs by binding to mineral surfaces [23, 24].

In our study, we found that exposure of HASMCs to HNs induced ROS generation, antioxidants partially attenuated the expression of Runx2, osterix and COL1, suggesting that oxidative stress was a necessary and sufficient condition for HN-induced osteogenic differentiation. Several studies have shown that oxidative stress promoted the VSMC differentiation process. For example, Gulinuer et al. [10] found that indoxyl sulfate increased ROS production and the expression of osteogenic proteins in VSMCs, and this effect could be blocked by antioxidants. Mathieu et al. [25] reported that ROS induction by palmitate enhanced the osteogenic differentiation of VSMCs. Although our study strongly suggests the implication of ROS in this transition, many downstream mechanisms may be involved in the final response. ROS could also activate endoplasmic reticulum stress, resulting in expression of the transcription factor XBP1, which binds to the Runx2 promoter and promotes Runx2 transcription [18].

We further examined the possible signaling involved in HN-induced differentiation of HASMCs. Herein we showed that HNs promoted osteogenic differentiation of HASMCs via the ERK pathway. This result is in agreement with previous findings suggesting that ERK activated the transcriptional activity of Runx2 in MC3T3-E1 cells [26, 27]. Moreover, we discovered that ROS was partially involved in this mechanism, because when we used antioxidants to inhibit ROS production, HN-induced ERK phosphorylation was suppressed.

In addition to being activated by ROS, the ERK pathway also responds to a variety of extracellular information, including ECM-integrin bind-

ing, mechanical stress, and growth factors [13]. In VSMCs, other mechanisms may also account for HN-enhanced phenotype switch via the ERK pathway. Firstly, ERK signaling was reported to regulate stress-induced osteogenic differentiation of MSCs [28, 29]. It is possible that HN serve as mechanical stimuli on VSMCs to trigger this transition. Secondly, ERK could mediate ECM-induced osteogenic differentiation [30, 31]. Given one important character of nanoparticles is absorption of proteins on their surface in media [32], we speculate that changing of ECM-integrin binding may interact with ERK signaling. In fact, Lu et al. [33], discovered that compared with adipose tissue-derived MSCs cultured on hydroxyapatite, cells seeded on HNs significantly induced expression of osteogenic genes via integrin-α2 and ERK signaling.

Taken together, our findings suggest that HNs could induce osteogenic differentiation of primary HASMCs and that this effect may occur partly via oxidative stress and ERK signaling. This study gives possible mechanisms for the effect of HNs and may help us better prevent and treat calcification in CKD patients.

Acknowledgements

This work was supported by National Nature Science Foundation Grant of China (81370864, 81670691) and the Grant from Science and Technology Commission of Shanghai Municipality (114119a5900). This manuscript has also been proofread and edited by a professional English editing company, BioScience Writers (BSW), located in Houston, Texas.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei Fang, Department of Nephrology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai 200127, People's Republic of China. Tel: 86-21-58752345; Fax: 86-21-58395057; E-mail: fangwei_sh@126.com

References

[1] Collins AJ, Foley RN, Gilbertson DT, Chen SC. United States renal data system public health surveillance of chronic kidney disease and end-stage renal disease. Kidney Int Suppl (2011) 2015; 5: 2-7.

[2] Shroff R, Long DA and Shanahan C. Mechanistic insights into vascular calcification in CKD. J Am Soc Nephrol 2013; 24: 179-89.

[3] Kendrick J and Chonchol M. The role of phosphorus in the development and progression of vascular calcification. Am J Kidney Dis 2011; 58: 826-34.

[4] Moe SM and Chen NX. Mechanisms of vascular calcification in chronic kidney disease. J Am Soc Nephrol 2008; 19: 213-6.

[5] Yamaguchi A. [Vascular calcification and bone-related factors]. Clin Calcium 2002; 12: 1078-83.

[6] Schlieper G, Aretz A, Verberckmoes SC, Krüger T, Behets GJ, Ghadimi R, Weirich TE, Rohrmann D, Langer S, Tordoir JH, Amann K, Westenfeld R, Brandenburg VM, D'Haese PC, Mayer J, Ketteler M, McKee MD, Floege J. Ultrastructural analysis of vascular calcifications in uremia. J Am Soc Nephrol 2010; 21: 689-96.

[7] Villa-Bellosta R, Millan A and Sorribas V. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. Am J Physiol Cell Physiol 2011; 300: C210-20.

[8] Sage AP, Lu J, Tintut Y, Demer LL. Hyperphosphatemia-induced nanocrystals upregulate the expression of bone morphogenetic protein-2 and osteopontin genes in mouse smooth muscle cells in vitro. Kidney Int 2011; 79: 414-22.

[9] Liu Y, Zhang L, Ni Z, Qian J, Fang W. Calcium phosphate crystals from uremic serum promote osteogenic differentiation in human aortic smooth muscle cells. Calcif Tissue Int 2016; 99: 543-555.

[10] Muteliefu G, Enomoto A, Jiang P, Takahashi M, Niwa T. Indoxyl sulphate induces oxidative stress and the expression of osteoblast-specific proteins in vascular smooth muscle cells. Nephrol Dial Transplant 2009; 24: 2051-8.

[11] Koleganova N, Piecha G, Ritz E, Schirmacher P, Müller A, Meyer HP, Gross ML. Arterial calcification in patients with chronic kidney disease. Nephrol Dial Transplant 2009; 24: 2488-96.

[12] Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, Frutkin A, Dichek D, Giachelli CM. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res 2009; 104: 733-41.

[13] Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol 2007; 176: 709-18.

[14] Murakami J, Ishii M, Suehiro F, Ishihata K, Nakamura N, Nishimura M. Vascular endothelial growth factor-C induces osteogenic differentiation of human mesenchymal stem cells

through the ERK and RUNX2 pathway. Biochem Biophys Res Commun 2017; 484: 710-718.
[15] Li CS, Zheng Z, Su XX, Wang F, Ling M, Zou M, Zhou H. Activation of the extracellular signal-regulated kinase signaling is critical for human umbilical cord mesenchymal stem cell osteogenic differentiation. Biomed Res Int 2016; 2016: 3764372.
[16] Kim JM, Lee JE, Ryu SH, Suh PG. Chlormadinone acetate promotes osteoblast differentiation of human mesenchymal stem cells through the ERK signaling pathway. Eur J Pharmacol 2014; 726: 1-8.
[17] Nadra I, Mason JC, Philippidis P, Florey O, Smythe CD, McCarthy GM, Landis RC, Haskard DO. Proinflammatory activation of macrophages by basic calcium phosphate crystals via protein kinase C and MAP kinase pathways: a vicious cycle of inflammation and arterial calcification? Circ Res 2005; 96: 1248-56.
[18] Liberman M, Johnson RC, Handy DE, Loscalzo J, Leopold JA. Bone morphogenetic protein-2 activates NADPH oxidase to increase endoplasmic reticulum stress and human coronary artery smooth muscle cell calcification. Biochem Biophys Res Commun 2011; 413: 436-41.
[19] Muteliefu G, Shimizu H, Enomoto A, Nishijima F, Takahashi M, Niwa T. Indoxyl sulfate promotes vascular smooth muscle cell senescence with upregulation of p53, p21, and prelamin a through oxidative stress. Am J Physiol Cell Physiol 2012; 303: C126-34.
[20] Ewence AE, Bootman M, Roderick HL, Skepper JN, McCarthy G, Epple M, Neumann M, Shanahan CM, Proudfoot D. Calcium phosphate crystals induce cell death in human vascular smooth muscle cells: a potential mechanism in atherosclerotic plaque destabilization. Circ Res 2008; 103: e28-34.
[21] Bostrom KI, Rajamannan NM and Towler DA. The regulation of valvular and vascular sclerosis by osteogenic morphogens. Circ Res 2011; 109: 564-77.
[22] Bear M, Butcher M and Shaughnessy SG. Oxidized low-density lipoprotein acts synergistically with beta-glycerophosphate to induce osteoblast differentiation in primary cultures of vascular smooth muscle cells. J Cell Biochem 2008; 105: 185-93.
[23] Jono S, Peinado C and Giachelli CM. Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. J Biol Chem 2000; 275: 20197-203.
[24] Viguet-Carrin S, Garnero P and Delmas PD. The role of collagen in bone strength. Osteoporos Int 2006; 17: 319-36.
[25] Brodeur MR, Bouvet C, Barrette M, Moreau P. Palmitic acid increases medial calcification by inducing oxidative stress. J Vasc Res 2013; 50: 430-41.
[26] Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res 2002; 17: 101-10.
[27] Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. J Biol Chem 2009; 284: 32533-43.
[28] Zhang P, Wu Y, Jiang Z, Jiang L, Fang B. Osteogenic response of mesenchymal stem cells to continuous mechanical strain is dependent on ERK1/2-Runx2 signaling. Int J Mol Med 2012; 29: 1083-9.
[29] Catalano MG, Marano F, Rinella L, de Girolamo L, Bosco O, Fortunati N, Berta L, Frairia R. Extracorporeal shockwaves (ESWs) enhance the osteogenic medium-induced differentiation of adipose-derived stem cells into osteoblast-like cells. J Tissue Eng Regen Med 2017; 11: 390-399.
[30] Viale-Bouroncle S, Gosau M and Morsczeck C. Laminin regulates the osteogenic differentiation of dental follicle cells via integrin-alpha2/-beta1 and the activation of the FAK/ERK signaling pathway. Cell Tissue Res 2014; 357: 345-54.
[31] Salasznyk RM, Klees RF, Hughlock MK, Plopper GE. ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. Cell Commun Adhes 2004; 11: 137-53.
[32] Verma A and Stellacci F. Effect of surface properties on nanoparticle-cell interactions. Small 2010; 6: 12-21.
[33] Lu Z, Roohani-Esfahani SI, Wang G, Zreiqat H. Bone biomimetic microenvironment induces osteogenic differentiation of adipose tissue-derived mesenchymal stem cells. Nanomedicine 2012; 8: 507-15.