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DNMT3A regulates differentiation of osteoblast and autophagy of vascular smooth muscle cells in vascular medial calcification induced by high phosphorus through ERK1/2 signaling

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Abstract

To investigate the effect of DNMT3A in vascular calcification (VC) induced by high phosphorus. The arterial tissues of 12 patients with end stage renal disease (ESRD) and VC and 12 patients with ESRD without VC were collected. Rat vascular smooth muscle cells (VSMCs) were divided into control group, high phosphorus (P) group, P + DMSO group, p-ERK1/2 inhibitor group, DNMT3A group and DNMT3A + P group and P + shRNA-DNMT3A group. Vascular calcification was evaluated by von kossa staining. Cell calcification was evaluated by alizarin red staining. The calcium content was assessed by calcium determination kit. The levels of DNMT3A, Runx2, LC3 and p-ERK1/2 were significantly up-regulated in CKD patients with VC in comparison with those in CKD patients without VC($p<0.05$). Moreover, the levels of SM22α and P62 were notably decreased in CKD patients with VC in comparison with those in CKD patients without VC($p<0.05$). Similar changes were observed in VSMCs induced by high phosphorus. Knock down of DNMT3A in VSMCs inhibited phenotypic transformation and induced autophagy, then reduced calcification($p<0.05$). Moreover, p-ERK1/2 level was downregulated by knock down of DNMT3A in comparison with the control group($p<0.05$). In conclusion, DNMT3A regulated high phosphorus induced vascular medial calcification via ERK1/2 signaling.

Keywords: DNMT3A; osteoblast differentiation; medial vascular calcification; autophagy; ERK1/2.

Practical Application: Our study suggested that DNMT3A was involved in the pathogenesis of medial VC, and DNMT3A regulated high phosphorus.

1 Introduction

Medial vascular calcification (VC) is one of the common complications in patients with chronic kidney disease (CKD) (Vervloet & Cozzolino, 2017). The prevalence of cardiovascular disease (CVD) in patients with CKD is substantially higher than that in the healthy controls, which is strongly associated with VC (Komatsu et al., 2014). In the past, VC was considered as a passive and degenerative process of calcium phosphate depositing in vessel wall (Liu, 2015). Recently, it has been reported that VC is a reversible and highly regulated process, which is associated with many factors and similar to osteogenesis (Peres & Pércio, 2014). Age, diabetes mellitus, chronic inflammation and hyperphosphatemia all play important roles in the development of VC (Bellasi et al., 2009; Román-García et al., 2009; Shigematsu et al., 2003).

The pathogenesis of medial VC is complicated. The phenotype transformation and autophagy of vascular smooth muscle cells (VSMCs) are both research focus. VSMCs, as a main component of vascular media, can transform into osteo-/chondrocytic-like cells in VC, which is manifested as up-regulation of osteogenic marker, Runx2, and down regulation of VSMC marker, SM22α (Smith, 2016). Autophagy is a highly controlled dynamic process, through which eukaryotic cells use lysosomes to degrade aging organelles and macromolecules substances (Klionsky, 2007; Lee et al., 2012; Levine & Klionsky, 2004). Several evidences suggest that phenotype transformation and autophagy play essential role in VC (Frauscher et al., 2018; Shroff & Shanahan, 2007). However, the molecular biological mechanisms are quite complicated and not fully understood.

DNA methylation is a stable epigenetic modifications. It occurs at the cytosine of the dinucleotide sequence CpG, which plays a key role in gene expression. DNA methyltransferases (DNMTs) occurs in promoter regions, regulating methylation of promoter sites, leading to down-regulation of the target gene (Pathania et al., 2015). DNA methyltransferases 3A (DNMT3A) is a member of the DNA methyltransferases family. It has been indicated that DNMT3A affects the formation of VC in patients with CKD by regulating VSMC phenotypic transformation (Chen et al., 2016). Furthermore, in cardiac fibrosis model, DNMT3A controls autophagy to modulate cardiac fibrosis progression (Zhao et al., 2018). DNMT3A are closely related to the phenotype switch and autophagy in VC. However, the molecular mechanisms underlying the regulation of DNMT3A in VC have not been clarified. Meanwhile, how DNMT3A affects

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differentiation of osteoblast and autophagy of VSMCs in VC remains unclear.

The relationship of DNMT3A and phenotypic transformation, autophagy in medial VC is unknown, and how DNMT3A leads to the development of VC is also currently unknown. It has been found that several pathways may be involved in the development of VC induced by DNMT3A upregulation, such as ERK1/2 signaling pathway (Voelkl et al., 2019). ERK1/2 pathway is a critical driver in VC development and bone homeostasis (Li et al., 2018). In this study, we aimed to examine the effect of DNMT3A on high phosphorus induced VC.

2 Materials and methods

2.1 Patients

This study was approved by the Ethics Committee of our hospital, Hebei Key Laboratory of Vascular Calcification in Kidney Disease, Hebei Clinical Research Center for Chronic Kidney Disease. Written informed consent was attained from each patient prior to this study. From January 2020 to August 2020, we enrolled 12 patients with end stage renal disease (ESRD) who diagnosed as VC according to von kossa staining (as VC group) and 12 patients with ESRD and non-VC (NVC group). Exclusion criteria: (1) the patients who were younger than 18 years old; (2) the patients with the artery that was too thin to retain tissue. Before anastomosis arterial and venous walls, arterial tissues including all layers of the artery wall were collected.

2.2 Materials and chemicals

Rabbit anti-Runx2 and rabbit anti-SM22a antibodies were obtained from Abcam (Cambridge, MA, USA). Rabbit anti-p-ERK1/2 (Thr202/Tyr204) and rabbit anti-ERK1/2 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-LC3 and rabbit anti-P62 antibodies were obtained from Proteintech Group, Inc. (Roawmont, IL, USA). Rabbit anti-DNMT3A antibody was obtained from Hua An Co. Ltd. (Hangzhou, Zhejiang, China). Rabbit anti-GAPDH was obtained from Affinity Biosciences Co. Ltd. (Changzhou, Jiangsu, China). PD0325901 was purchased from MCE Co. Ltd. (Monmouth Junction, NJ, USA). The small hairpin RNA (shRNA) oligos of rat DNMT3A and negative control (NC) were purchased from GenePharma Biotechnology Co. Ltd. (Shanghai, China). Lipofectamine 3000 were purchased from Invitrogen Co. Ltd. (Carlsbad, CA, USA).

2.3 Cell culture, groups and treatment

Rat VSMCs were purchase from Shanghai Baili Biotechnology Co., Ltd. (Shanghai, China) and cultured using the method as previously described (Xu et al., 2015). VSMCs were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) for 2 days, 7 days and 9 days. To investigate the effect of high phosphorus on VC and DNMT3A, phenotype transformation and autophagy expression, VSMCs were divided into the normal control (N) group, the high phosphorus induced VC group (P; VSMCs were cultured with 10 mmol/L β-glycerophosphate stimulation). To identify the effect of DNMT3A on phenotype switch of VSMCs and autophagy in VC, VSMCs were divided into four groups: the normal control group (N), the high phosphorus induced VC group (P), negative control shRNA group (P+NC), DNMT3A shRNA group (P+shRNA-DNMT3A). To explore how ERK1/2 regulates phenotype transformation and autophagy in VC, VSMCs were divided into four groups: the normal control group (N), the high phosphorus induced VC group (P), DMSO control group (P+DMSO) and PD0325901 group (PD0325901, as a selective P-ERK1/2 inhibitor, was added to high phosphate induced VSMCs at a final concentration of 10 μm for 2 days).

2.4 Cells transfection

DNMT3A-shRNA and shRNA-negative control (NC) plasmids were transfected in VSMCs according to lipofectamine 3000 instructions. A 2 μL plasmid and 6 μL P3000 were mixed with 125 μL Opi-MEM. 5 μL lip3000 was added into 125 μL Opi-MEM. Then the mixture was added to VSMCs, which was cultured in 6 well plates after 5 min. After 48 h transfection, the non-transfected or transfected cells were collected for further experiments.

2.5 Immunohistochemistry staining

Immunohistochemistry staining was performed as previously described (Zhang et al., 2018). The slices of arterial tissues were processed with high-pressure thermal remediation. The slices were blocked with 8% goat serum for 25 min and incubated with DNMT3A, Runx2, SM22a, LC3, P-ERK or P62 primary antibodies overnight at 4 °C. Afterwards, the secondary antibody was incubated for 1 h at 37 °C. Images were analyzed by Image-Pro Plus (Media Cybernetics, Bethesda, MD), the integrated option density (IOD) of positive regions was analyzed.

2.6 Von kossa staining

Von kossa staining was used to assess the pathological changes of VC in arterial tissues. Briefly, the slices of arterial tissues were stained with 5% silver nitrate solution, irradiated with ultraviolet rays for 50 min. Then the slices were soaked in 5% sodium thiosulfate solution for 5 min. Afterwards, the slices were soaked in 0.1% hematoxylin-eosin for 2 min. The images were observed under a fluorescence microscope. Black particles were deposited in calcification regions.

2.7 Alizarin red staining

Alizarin red staining was performed to evaluate the calcification in VSMCs as previous described (Yang et al., 2009). VSMCs were fixed in 95% ethyl alcohol for 30 min, followed by incubation with 0.1% Alizarin red solution at 37 °C for 40 min. Alizarin red staining were examined and photographed with microscope.

2.8 Calcium content

The calcium content in VSMCs was measured by using calcium assay kit (Zhongsheng Beikong Biotechnology Co., Ltd, Beijing, China) according to the manufacturer's instructions.

Protein concentrations were detected by bicinchoninic acid (BCA) protein assay kit (Solarbio Science & Technology Company Co., Ltd, Beijing, China). The calcium content was normalized to the amount of the protein content.

2.9 Real-time quantitative polymerase chain reaction (RT-qPCR)

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to analyze the mRNA expression of DNMT3A, Runx2, GAPDH and SM22α in VSMCs. The cells were lysed using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). Total RNA were reverse transcribed into cDNA using Superscript TM III Reverse Transcriptase (Invitrogen Carlsbad, CA, USA). The relative mRNAs expression levels were calculated using $2^{-\Delta\Delta CT}$ method, and normalized with GAPDH. The primer sequences of DNMT3A, Runx2, SM22α and GAPDH were shown in Table 1.

2.10 Western blotting analysis

Western blotting was performed as previously described (Xu et al., 2015). Proteins in VSMCs were extracted and quantified by BCA assay kit. After electrophoresis on 12% SDS-PAGE gels, proteins were transferred to nitrocellulose membrane (Pall Co., NY, USA). The blots were incubated overnight at 4°C with GAPDH, DNMT3A, Runx2, SM22α, P-ERK1/2, ERK1/2, LC3 and P62 primary antibodies. The membranes were incubated with the secondary antibody at room temperature for 2 h and detected with the Enhanced Chemiluminescence Kit (Amersham, Piscataway, NJ, USA). The band intensity was analyzed by using Image J software.

2.11 Statistical analysis

All experiments were repeated three times with the same sample. All data were analyzed by SPSS software version 23.0 (International Business Machines, corp., Armonk, NY, USA). Data were expressed as mean ± standard deviation(SD). Significant differences between groups were assessed by One-way analysis of variance (ANOVA). $p<0.05$ was considered statistically significant.

3 Results

3.1 Expression of DNMT3A, osteoblast differentiation marker, autophagy marker and p-ERK1/2 in patients with VC

Among patients with ESRD, 12 patients with VC and 12 matched patients without VC were recruited according to von kossa staining. The characteristics and reasons for the VC group and NVC group were shown in Table 2 and Table 3. Compared to NVC group, serum phosphorus was higher in VC group ($p<0.05$). There were no differences in other characteristics between the two groups ($p>0.05$). Furthermore, patients in VC group had higher DNMT3A expression ($p<0.05$) (Figure 1A, 1E). We further investigated the relevant markers of above mechanisms. There was

Table 1. Primers of human DNMT3A, GAPDH.

	Forward primer	Reverse primer
DNMT3A	CTCTGGCGGAGGTCGGGA	TCTGTCAGCCTGTGGGTGGG
Runx2	GCTGGAGTGATGTGGTTTTCT	CTATGAAGCCTGGCGATTT
GAPDH	GGGAAACTGTGGCGTGAT	GAGTGGGTGTCGCTGTTGA

Table 2. Primers of rat DNMT3A, Runx2, SM22a, GAPDH.

	Forward primer	Reverse primer
DNMT3A	GGCCCATTCGATCTGGTGA	CTTGGCTATTCTGCCGTGTTC
Runx2	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
SM22a	AACAGCCTGTACCCTGATGG	CACTGGGCAGACAGTCAGAA
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

Table 3. Characteristics of VC and no VC ESRD patients.

	All patients	VC group	No VC group	P
	N=24	N=12	N=12	
Age, years	73.54 ± 7.85	76.50 ± 8.08	70.58 ± 6.67	.064
Male, n (%)	12 (50.0)	7 (58.3)	5 (41.7)	.219
BMI, kg/m^2	23.63 ± 3.87	24.63 ± 4.73	22.62 ± 2.60	.212
Reason for AVF				
T2DM	9(37.5)	5(41.7)	4(33.3)	.673
HT	5(20.8)	2(16.7)	3(25.0)	.614
GN	5(20.8)	2(16.7)	3(25.0)	.614
Others	5(20.8)	3(25.0)	2(16.7)	.614
P, mmol/L	1.79 ± 0.69	2.03 ± 0.81	1.56 ± 0.47	**.034**

VC = vascular calcification; ESRD = end stage renal disease; BMI = body mass index; AVF = arteriovenous fistula; T2DM = type 2 diabetes mellitus; HT = hypertension disease; GN = glomerulonephritis; N=No VC group; P=VC group.

high Runx2 expression in the VC group in comparison with NVC group, while there was decreased SM22α expression in the VC group in comparison with NVC group (p<0.05) (Figure 1B, 1E). Meanwhile, LC3 concentration, a autophagy marker, was increased in the VC group, but P62 level was decreased in the VC group compared with those in NVC group (p<0.05) (Figure 1C, 1E). We also observed high p-ERK1/2 expression in the slices of calcified arterial tissues (p<0.05) (Figure 1D, 1E).

3.2 Expression of DNMT3A, osteoblast differentiation marker, autophagy marker, ERK1/2 in β-glycerophosphate induced cell calcification

Consistent with our previous studies, we investigated cell calcification and calcium content. More calcified nodules and calcium content were found in VSMCs after 9-day culture (Figure 2A, 2B). In β-glycerophosphate stimulated group (P group), the DNMT3A

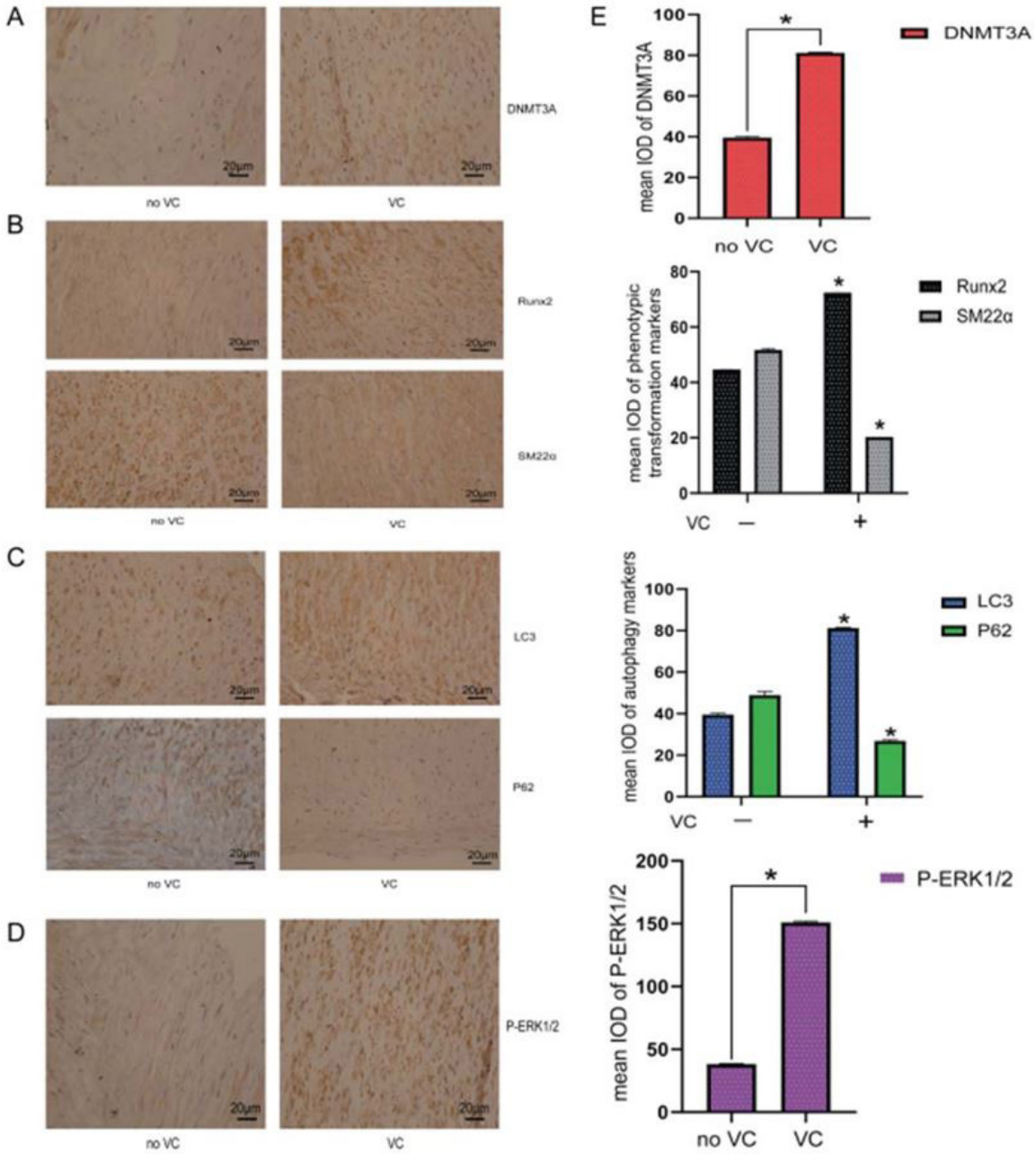

Figure 1. Difference expression of DNMT3A, phenotypic transformation markers, autophagy markers, p-ERK1/2 in no VC and VC groups. (A) Expression of DNMT3A using Immunohistochemistry Staining in tissues; (B) Expression of phenotypic transformation markers (Runx2, SM22α) using Immunohistochemistry Staining in tissues; (C) Expression of autophagy markers (LC3, P62) using Immunohistochemistry Staining in tissues; (D) Expression of P-ERK1/2 using Immunohistochemistry Staining in tissues; (E) Mean IOD of DNMT3A, phenotypic transformation markers, autophagy markers, p-ERK1/2 in arterial tissues. NVC group vs VC group, *p<0.05.

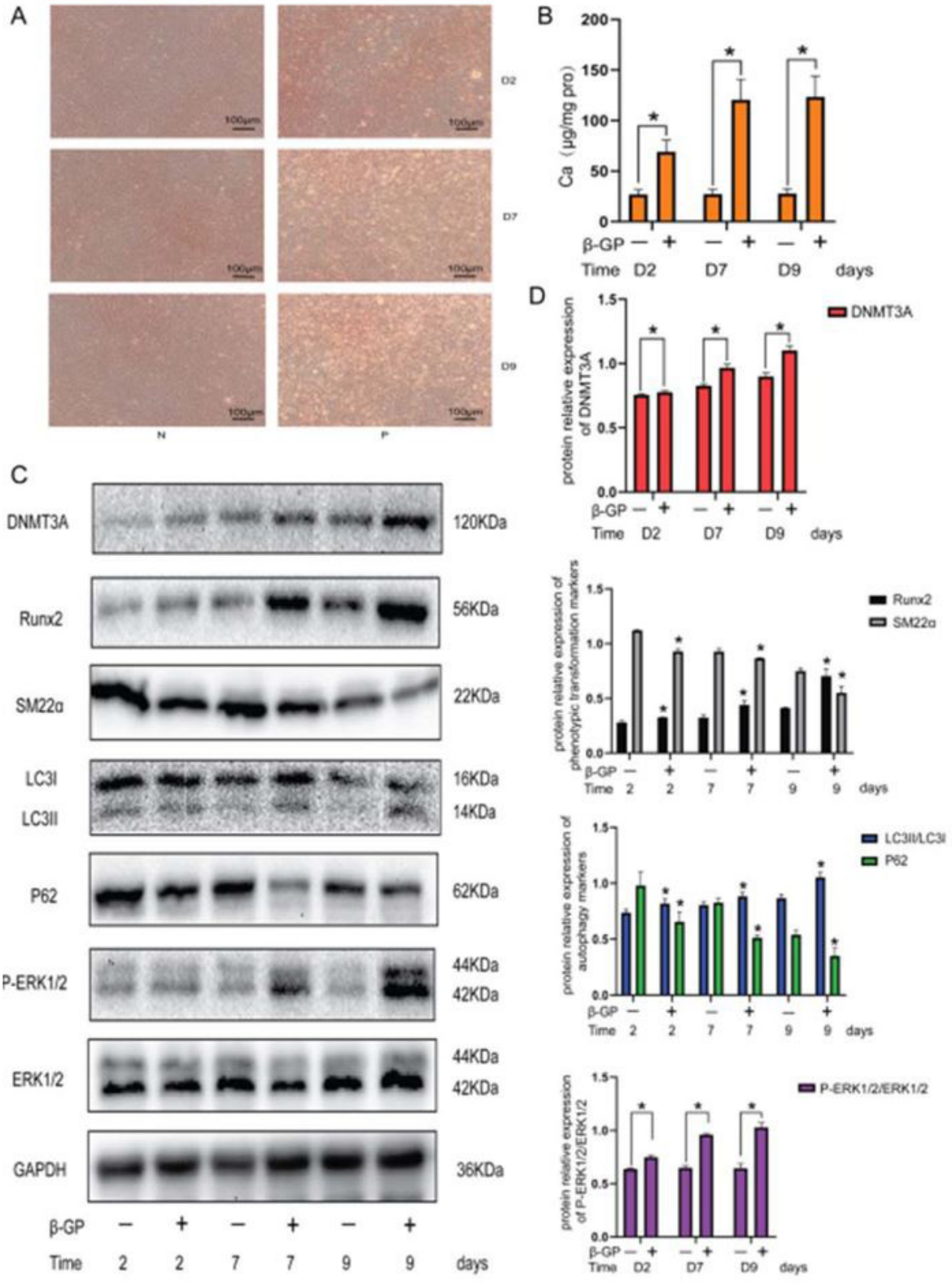

Figure 2. The protein expression of DNMT3A, phenotypic transformation markers, autophagy markers, ERK1/2 signaling in normal group (N group) and β-glycerophosphate-induced group (P group) at rat VSMCs. (A) Alizarin Red Staining in N and P group after 2 days,7 days,9 days of cell culture; (B) Calcium content in N and P group after 2 days, 7 days, 9 days of cell culture; (C) DNMT3A, phenotypic transformation markers (SM22α, Runx2), autophagy markers (LC3, P62), ERK1/2 signaling (P-ERK1/2, ERK1/2) protein relative expression were analyzed by western blot; (D) Analysis of DNMT3A, phenotypic transformation markers (SM22α, Runx2), autophagy markers (LC3II/LC3I ratio, P62), P-ERK1/2/ERK1/2 protein expression using Image J software. N group vs P group, *$p<0.05$.

protein level was notably increased (p<0.05) (Figure 2C, 2D). There were a significant up-regulated Runx2 protein expression and decreased SM22α protein expression in β-glycerophosphate induced VSMCs (p<0.05) (Figure 2C, 2D). The mRNA expression levels of DNMT3A, Runx2 and SM22α were consistent with the results of western blot (Figure 3A, 3B). This manifested that both DNMT3A and osteoblast differentiation of VSMCs were up-regulated in P group. Moreover, the protein expression of autophagy markers, including LC3, P62 and P-ERK1/2, were observed by western blot. Compared to normal group (N group), the ratio of LC3II/LC3I, p-ERK1/2/ERK1/2 were higher, but the P62 protein expression level was decreased, especially after 9 days of culture (Figure 2C, 2D). Those results suggested that autophagy and ERK1/2 signaling were significantly increased in β-glycerophosphate induced VSMCs.

3.3 Down-regulation of DNMT3A reduced osteoblast differentiation, p-ERK1/2 and induced autophagy while inhibiting calcification in β-glycerophosphate induced VSMCs

To confirm the effect of DNMT3A on VC and how DNMT3A regulates phenotypic transformation and autophagy, we transfected shRNA-DNMT3A into VSMCs. Compared with the P+NC group, the calcium content was decreased in the shRNA-3A group (Figure 4A, 4B). The protein expression levels of DNMT3A and Runx2 were notably down-regulated, while the SM22α protein expression level was markedly up-regulated (Figure 4C, 4D). The mRNA expression levels of DNMT3A, Runx2 and SM22α were consistent with the results of western blot (Figure 5A, 5B). It was verified that DNMT3A made an effect on phenotypic transformation of VSMCs. Moreover, the ratio of LC3II/LC3I was increased, but the P62 expression level was decreased (Figure 4C, 4D). After transfected shRNA-DNMT3A, the protein expression levels of p-ERK1/2 and ERK1/2 were significantly down-regulated (Figure 4C, 4D). This demonstrated that ERK1/2 pathway was regulated by DNMT3A, and ERK1/2 signaling was involved in phenotypic transformation and autophagy regulated by DNMT3A, resulting in VSMCs calcification.

3.4 Down-regulation of p-ERK1/2 reduced osteoblast differentiation and induced autophagy in β-glycerophosphate induced VSMCs

To further identify the effect of ERK signaling on osteoblast differentiation and autophagy of VSMCs, we treated cells with PDO325901, a common used inhibitor of p-ERK1/2. Accompany with inhibiting of ERK signaling, PD0325901 also reduced osteoblast differentiation, manifesting decreased Runx2 protein expression and increased SM22α expression. However, after treated with PD0325901 for 48 h, there was a significant increase in the ratio of LC3II/LC3I, a marked decrease in P62 expression (Figure 6A, 6B).

4 Discussion

In this study, we found high phosphorus level in ESRD patient with VC, and calcification of VSMCs were also induced by hyperphosphorus. Osteoblast differentiation and autophagy were main mechanisms of medial calcification *in vitro* and *in vivo*. DNMT3A was upregulated in VC *in vitro* and *in vivo*. DNMT3A exacerbated VC by regulating phenotypic transformation and autophagy. DNMT3A regulated phenotype switch and autophagy in calcification in the VSMCs through ERK1/2 pathway.

VC is involved in several risk factors. Previous studies have shown that hyperphosphorus was closely related to VC progress and mortality in general and CKD patients (Shang et al., 2017; Ritter & Slatopolsky, 2016; Selamet et al., 2016; Eddington et al., 2010; Dhingra et al., 2007), and hyperphosphorus is a key risk factor for regulating VC in CKD patients (Kendrick & Chonchol, 2011). Consistent with previous studies, we found that phosphorus in CKD patients with VC was significantly higher than those without VC, and phosphorus also stimulated calcification in VSMCs in a time manner. The main mechanism by which phosphate induces VC in CKD includes promoting the

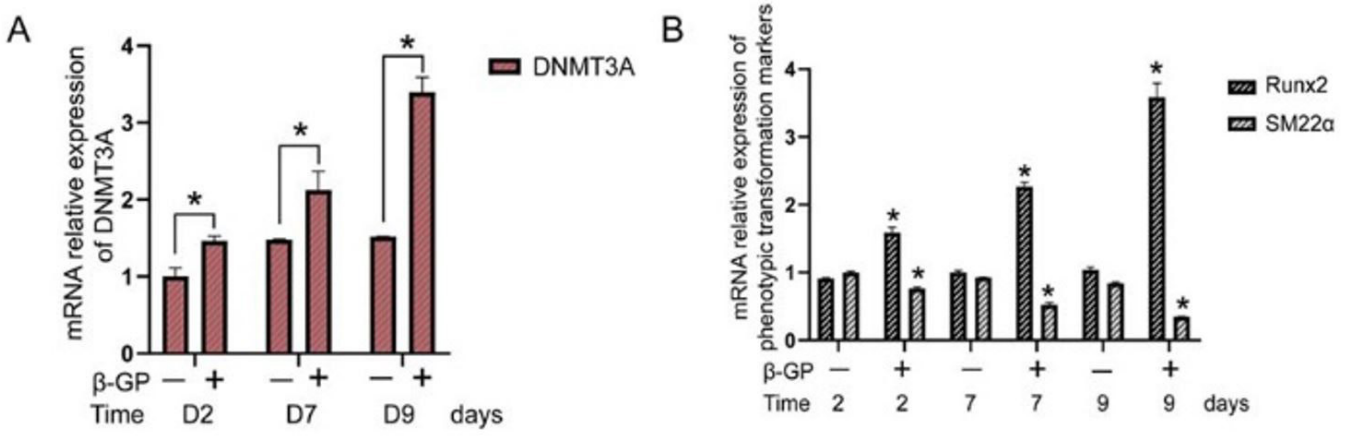

Figure 3. The expression of DNMT3A, phenotypic transformation markers by RT- qPCR. (A) DNMT3A expression were evaluated by RT-qPCR; (B) phenotypic transformation markers (SM22α, Runx2) expression were evaluated by RT-qPCR. N group VS P group, *p<0.05.

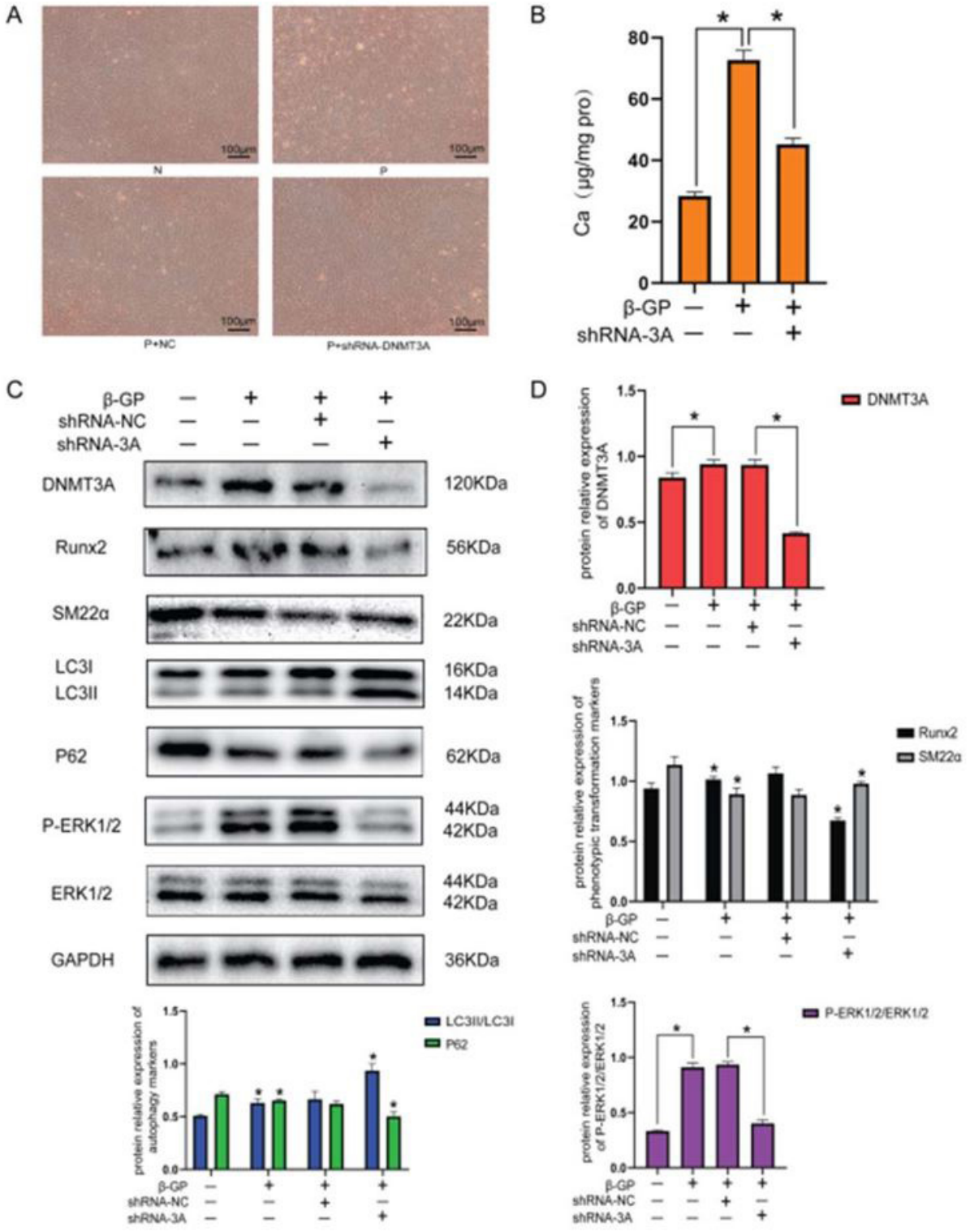

Figure 4. After transfected shRNA-DNMT3A,VSMCS were randomly divided into four groups: the normal group (N group), β-glycerophosphate induced group (P group), P group + shRNA-negative control group (P+shRNA-NC group), P+shRNA-DNMT3A (P+shRNA-3Agroup). Knockdown of DNMT3A by transfecting shRNA-DNMT3A induced VC in P group by inhibiting ERK signaling way, VSMCs phenotypic transformation and promoting autophagy. (A) Alizarin Red Staining was used to measure calcified nodules; (B) different calcium content in N, P, P+shRNA-NC, P+shRNA-3A groups; (C) Total protein was isolated from 48 h cultures after transferred shRNA-DNMT3A, DNMT3A, phenotypic transformation markers (Runx2, SM22α), autophagy markers (LC3, P62), ERK1/2 signaling (P-ERK1/2, ERK1/2) protein expression were analyzed by western blot; (D) DNMT3A, phenotypic transformation markers (SM22α, Runx2), autophagy markers (LC3II/LC3I ratio, P62), P-ERK1/2/ERK1/2 protein relative expression were evaluated by Image J software. N group vs P group, P+shRNA-NC group vs P+shRNA-3A group, *p<0.05.

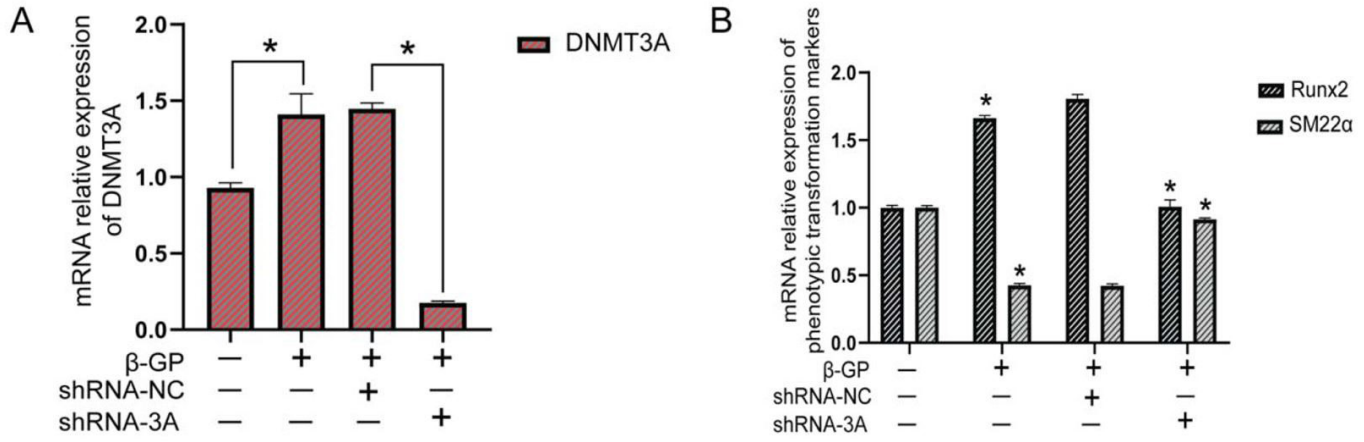

Figure 5. After transfected shRNA-DNMT3A, total RNA was isolated from 48 h cell cultures. (A) DNMT3A mRNA expression were evaluated by RT-qPCR; (B) phenotypic transformation markers (SM22α, Runx2) expression were evaluated by RT-qPCR. N group vs P group, P+shRNA-NC group VS P+shRNA-3A group, *p<0.05.

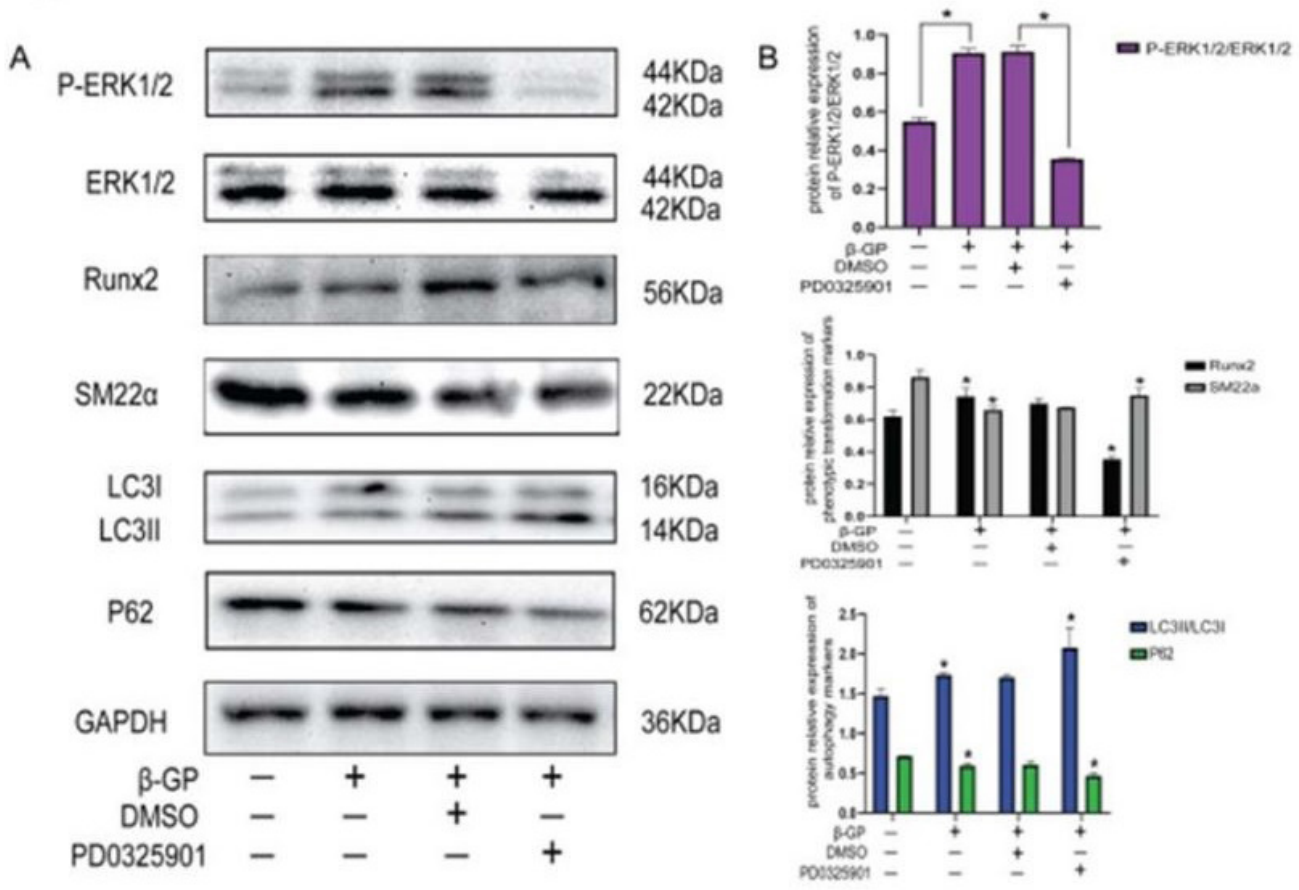

Figure 6. After treated with PD0325901,VSMCS were randomly divided into four groups: the normal group (N group), β-glycerophosphate induced group(P group), P group+ DMSO(P+DMSO group), P+PD0325901 group. Inhibiting ERK signaling way played a role in osteoblast differentiation and autophagy. (A) Total protein was isolated from 48 h cultures after treatment of PD0325901, ERK1/2 signaling (P-ERK1/2, ERK1/2), phenotypic transformation markers (Runx2, SM22α), autophagy markers (LC3, P62) protein expression were analyzed by western blot; (B) Analysis of P-ERK1/2/ERK1/2 ratio, phenotypic transformation markers (SM22α, Runx2), autophagy markers (LC3II/LC3I ratio, P62) protein relative expression were evaluated by Image J software. N group vs P group, P+DMSO group vs P+PD0325901 group, *p<0.05.

osteochondrogenic phenotype change of VSMCs (Giachelli et al., 2005; Jono et al., 2000). Runx2, as an osteoblast marker, was proved to be associated with osteoblast differentiation (Cai et al., 2013). In this study, increased Runx2 expression and decreased SM22α expression were found in the VC group. The cell model of β-glycerophosphate induced VSMCs was used to elucidate the pathologic process of calcification, and similar changes of Runx2 and SM22α were also observed. These data indicated that osteoblast-like transformation of VSMCs was a critical process and mechanism of calcification.

Then, we further investigated the expression and effect of autophagy on patients with medial VC. In previous studies, there were several mechanisms by which autophagy counteracted phosphate induced VC, including reducing matrix vesicle release (Dai et al., 2013), reducing oxidative stress (Sudo et al., 2015), and regulating miRNA expression (Xu et al., 2019). In our study, in the VC group or in the calcium of VSMCs, autophagy was upregulated. Increased autophagy level might protect against the development of VC. However, its protective effect is insufficient. It has been suggested that such counter-regulatory vascular protective mechanism increased autophagy, which is consist with another study (Frauscher et al., 2018). Knock-down of DNMT3A increased autophagy, thereby reducing medial VC, which also demonstrated the protective role of autophagy in VC.

DNA methylation is tightly linked to gene expression. DNMT3A, as one of DNMT proteins, promotes the methylation of DNA and affects gene expression (Xie et al., 1999). Furthermore, DNA methylation abnormalities are often observed in various diseases (Dees et al., 2014). Chen et al. (2016) reported that DNMT3A in calcified human aortic smooth muscle cells treated with indoxyl sulfate was significantly increased and inhibition of DNA methyltransferases by 5-az-a-2'-deoxycytidine caused demethylation of the klotho gene and decreased VC. As indicated in our study, DNMT3A expression was upregulated in CKD patients with VC and calcification in the VSMCs. Medial VC induced by β-glycerophosphate was greatly reduced by shRNA-DNMT3A. Therefore, DNMT3A played a significantly role in promoting VC.

As DNMT3A played an important role in regulating VC, the mechanisms of phenotype switch and autophagy were elucidated. We further explored the relationship between DNMT3A and osteoblast differentiation, and DNMT3A and autophagy in VC. SM22α promoter methylation in VSMCs was reported to be related to high phosphate induced calcification (Montes de Oca et al., 2010). In this study, we found that osteoblast differentiation was down-regulated after transfected with shRNA-DNMT3A, then medial VC was inhibited. To our knowledge, there was few study that investigated the relationship between DNMT3A and autophagy in VC. As our data showed, autophagy was upregulated, which attenuated VC after transfected with shRNA-DNMT3A. Therefore, we inferred that DNMT3A affected autophagy in VSMCs calcification. In cancer models, autophagy has multiple genes regulated by DNA methylation (Bhol et al., 2020). Perhaps, DNMT3A regulates autophagic and associated protein involved in medial VC, although the mechanisms are still unclear and need further study.

On the basis of the above findings, we then evaluated the ERK1/2 signaling in VC. ERK1/2 pathway mediates the functional effect of VC (Voelkl et al., 2019; Blanc et al., 2004; Liu et al., 2014). We further confirmed that ERK1/2 pathway was activated in calcification and acted to regulate osteoblast-like transformation in VSMCs and autophagy in medial VC. We treated VSMC with PD0325901, an inhibitor of p-ERK1/2 pathway. Exactly, inhibiting of p-ERK1/2 *in vivo* resulted in the upregulation of autophagy and the decline of phenotype switch in medial calcification. Moreover, ERK1/2 signaling was regulated by DNMT3A. After treatment with shRNA-DNMT3A, ERK1/2 pathway was suppressed. Taken together, our results revealed a direct impact of DNMT3A-ERK axis on the pathogenesis of osteoblast differentiation of VSMCs and autophagy in medial VC. However, DNMT3A usually silences gene expression in this study. When DNMT3A was knocked down, the ERK expression was decreased. However, the reason for the conflicting results remains unclear. Zhao X reported that DNMT3A controlled miRNA-200b in cardiac fibroblast (Zhao et al., 2018). Therefore, DNMT3A may regulate miRNAs or lncRNAs and then these non-coding RNA up-regulate ERK pathway. Further study will be essential to clearly identify the mechanisms.

5 Conclusion

In conclusion, our study suggested that DNMT3A was involved in the pathogenesis of medial VC, and DNMT3A regulated high phosphorus induced osteoblast differentiation in the VSMCs and autophagy in vascular medial calcification through ERK1/2 pathway.

Abbreviations

VC: vascular calcification; ESRD: end stage renal disease; VSMCs: vascular smooth muscle cells; Runx2: runt-related transcription factor 2; SM22α: smooth muscle 22 α; LC3: light chain 3; CKD: chronic kidney disease; CVD: cardiovascular disease; DNMTs: DNA methyltransferases; DNMT3A: DNA methyltransferases 3A; shRNA: small hairpin RNA; NC: negative control; DMEM: Dulbecco's modified Eagle medium; FBS: fetal bovine serum; IOD: integrated option density; BCA: bicinchoninic acid; RT-qPCR: Real-time Quantitative Polymerase Chain Reaction; SD: standard deviation; ANOVA: One-way analysis of variance.

Ethical approval

This study was approved by the Ethics Committee of the The Fourth Hospital of Hebei Medical University, Hebei Key Laboratory of Vascular Calcification in Kidney Disease, Hebei Clinical Research Center for Chronic Kidney Disease [2020-257-01(Z)]. Written informed consent was attained from each patient prior to this study.

Conflict of interest

There are no potential conflicts of interest to disclose.

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Author contributions

XYM is resposible for the study concepts, literature research, manuscript preparation & editing; MJC is resposible for the study concepts & design, experimental studies, manuscript review; JJJ is resposible for the data analysis, manuscript review; YLB is resposible for the definition of intellectual content, data acquisition, manuscript review; HRZ is resposible for the definition of intellectual content, data analysis, statistical analysis, manuscript review; LH is resposible for the definition of intellectual content, data analysis, statistical analysis, manuscript review; WZ is resposible for the definition of intellectual content, data analysis, statistical analysis, manuscript review; DXZ is resposible for the definition of intellectual content, experimental studies, statistical analysis, manuscript review; SLZ is resposible for the study concepts, definition of intellectual content, clinical studies, manuscript review; JSX is resposible for the guarantor of integrity of the entire study, definition of intellectual content, manuscript review. All authors read and approved the final manuscript.

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