Experimental Cell Research 345 (2016) 206–217

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

WNT/β-catenin signaling promotes VSMCs to osteogenic transdifferentiation and calcification through directly modulating Runx2 gene expression

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ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form
10 June 2016
Accepted 10 June 2016
Available online 16 June 2016

Keywords:
WNT/β-catenin signaling
Runx2
Vascular smooth muscle cells
Osteogenic transdifferentiation
High-phosphate
Arterial medial calcification

ABSTRACT

Arterial medial calcification (AMC) is prevalent in patients with chronic kidney disease (CKD) and contributes to elevated risk of cardiovascular events and mortality. Vascular smooth muscle cells (VSMCs) to osteogenic transdifferentiation (VOT) in a high-phosphate environment is involved in the pathogenesis of AMC in CKD. WNT/β-catenin signaling is indicated to play a crucial role in osteogenesis via promoting Runx2 expression in osteoprogenitor cells, however, its role in Runx2 regulation and VOT remains incompletely clarified. In this study, Runx2 was induced and β-catenin was activated by high-phosphate in VSMCs. Two forms of active β-catenin, dephosphorylated on Ser37/Thr41 and phosphorylated on Ser675 sites, were upregulated by high-phosphate. Activation of β-catenin, through ectopic expression of stabilized β-catenin, inhibition of GSK-3β, or WNT-3A protein, induced Runx2 expression, whereas blockade of WNT/β-catenin signaling with Porcupine (PORCN) inhibitor or Dickkopf-1 (DKK1) protein inhibited Runx2 induction by high-phosphate. WNT-3A promoted osteocalcin expression and calcium deposition in VSMCs, whereas DKK1 ameliorated calcification of VSMCs induced by high-phosphate. Two functional T cell factor (TCF)/lymphoid enhancer-binding factor binding sites were identified in the promoter region of *Runx2* gene in VSMCs, which interacted with TCF upon β-catenin activation. Site-directed mutation of each of them attenuated Runx2 response to β-catenin, and deletion or destruction of both of them completely abolished this responsiveness. In the aortic tunica media of rats with chronic renal failure, followed by AMC, Runx2 and β-catenin was induced, and the Runx2 mRNA level was positively associated with the abundance of phosphorylated β-catenin (Ser675). Collectively, our study suggested that high-phosphate may activate WNT/β-catenin signaling through different pathways, and the activated WNT/β-catenin signaling, through direct downstream target *Runx2*, could play an important role in promoting VOT and AMC.

1. Introduction

Arterial medial calcification (AMC), also known as Mönckeberg's medial sclerosis, is one of major pathological types of vascular calcification, which is highly prevalent in patients with chronic kidney disease (CKD) [1–4]. Hyperphosphatemia, a common clinical feature in patients with CKD and subsequent end stage renal disease, is germane to the development and progression of AMC and is identified as a major risk factor which is closely related to cardiovascular morbidity and mortality [5–7]. Although the pathogenesis of AMC hasn't as yet been elucidated, the genesis and development process is believed to replicate skeletal bone formation [8]. Exposure of vascular smooth muscle cells (VSMCs) to a high-phosphate environment, resembling the pathophysiological state in CKD patients, has been demonstrated to initiate their transdifferentiation to osteoblast-like cells and perform a cellular program which regulates the process of bone matrix deposition in vessel wall [9,10].

A number of factors have been evidenced to be crucial for osteogenesis and bone remodeling. Among them, Runx2 and WNT/β-catenin signaling are especially indispensible, since they are functionally connected elements of a pathway that is necessary for the commencement of osteoblast differentiation [11–13]. Belonging to the transcription factor family of *runt* homology domain, Runx2 is an acknowledged master transcription factor in osteoblast precursor cells and has been recognized as the earliest

Abbreviations: VSMCs, vascular smooth muscle cells; AMC, arterial medial calcification; β-GP, β-glycerophosphoricacid; LRP, low density lipoprotein receptor-related protein; GSK, glycogen synthase kinase; PORCN, Porcupine

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http://dx.doi.org/10.1016/j.yexcr.2016.06.007

marker of osteoblasts [11–13]. The signal transduction of WNT proteins is through plasma membrane Frizzled (FZD) receptors and coreceptors, members of low density lipoprotein receptor-related protein-5/6 (LRP5/6). Once coupling with their receptors/coreceptors, WNTs evoke cascaded downstream signaling events, resulting in the dephosphorylation and stabilization of β-catenin, which leads to β-catenin translocation into the nucleus, where it interacts with its DNA-binding partner, *i.e.* T cell factor (TCF)/lymphoid enhancer-binding factor (LEF), to initiate the transcription of WNT targeted genes [14–16]. In this WNT/β-catenin signaling pathway, it is the inhibition of glycogen synthase kinase 3 (GSK3) rendering a decreased phosphorylation of β-catenin on Ser33/Ser37/Thr41 sites and a reduced degradation of β-catenin, that is responsible for the stabilization and activation of β-catenin. In addition, phosphorylation of β-catenin on Ser675 site by protein kinase A (PKA) or p21-activated kinase 1 (PAK1) also leads to β-catenin activation [17–19]. It is well known that WNT/β-catenin signaling is essential for the osteogenic differentiation of pluripotent mesenchymal cells. Activation of WNT/β-catenin signaling by pro-osteogenic factors leads to Runx2 induction, which in turn, regulates pivotal processes essential for osteoblast differentiation and phenotypic characterization through governing the expression of several bone-related proteins, such as osterix, osteocalcin, and sclerostin [8,12,13,20,21].

WNT/β-catenin signaling has been demonstrated to be involved in the high-phosphate-induced VSMCs osteogenic transdifferentiation (VOT) and the development of AMC [22–27]. Activation of the Runx2 promoter through a TCF site was observed in mouse osteoprogenitor cells *in vitro* [12]. However, the cell-specific transcriptional mechanism through which WNT/β-catenin signaling promotes Runx2 expression in VSMCs and VOT has yet to be identified.

In this study, we demonstrate that Runx2 is induced and β-catenin is activated in the VSMCs in a high-phosphate environment both *in vitro* and *in vivo*. High-phosphate may promote WNT secretion, and then activate β-catenin via different pathways. Activation of WNT/β-catenin signaling, through ectopic expression of stabilized β-catenin, inhibition of GSK-3β, or WNT-3A protein, induces Runx2 expression in VSMCs. WNT-3A also promotes osteocalcin induction and calcification of VSMCs. Blockade of WNT/β-catenin signaling by DKK1 inhibits Runx2 induction and calcium deposition stimulated by high-phosphate in VSMCs. We further demonstrate that WNT/β-catenin signaling is responsible for Runx2 induction in VSMCs. Our studies identify Runx2 as a direct downstream target of WNT/β-catenin signaling during the high-phosphate-triggered VOT and unravel a potential mechanism underlying this signal pathway in the pathogenesis of AMC in CKD patients.

2. Material and methods

2.1. Cell culture and treatment

Primary rat aortic smooth muscle cells (*p*-VSMCs) were isolated and cultured as described previously [28]. Briefly, prepubertal male Sprague-Dawley rats purchased from Shanghai Experimental Animal Center (Shanghai, China) were anesthetized by sodium pentobarbital, and exsanguinated for euthanasia, and the arterial segments were acquired. After the inner portion was separated out, the media was cut into ~1-mm^2 sections and placed in a cell culture dish with Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 20% fetal bovine serum (FBS) and 10 mM sodium pyruvate. Cells migrated from the explants were cultured in DMEM with 15% FBS. P-VSMCs between passages 3 and 8 were used in experiments. For high-phosphate treatment, the cells were seeded at ~70% confluence in complete medium with 15% FBS for 24 h and then serum-starved for 16 h, followed by incubation with 10 mM β-glycerophosphoricacid (β-GP) (Sigma-Aldrich, St Louis, MO) for various periods of time. For some experiments, after serum starvation, cells were either incubated with 10 ng/ml recombinant WNT-3A (cat. no. 1324-WN010, R&D Systems, Minneapolis, MN) for various periods of time or different concentration as indicated for 24 h; or pretreated with 1 nM LGK794 (porcupine inhibitor, cat. no. HY-17545, Medchemexpress), 3 μM IM-12 (GSK-3β inhibitor, cat.no. s7566, Selleck), or 100 ng/ml recombinant DKK1 protein (cat.no. 4010-DK-010, R&D Systems) for 30 min, followed by incubation with or without 10 mM β-GP for 24 h.

Rat thoracic aorta smooth muscle cell line (L-VSMC, A7r5) was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), cultured in DMEM with 10% FBS, and used for plasmid transfection.

2.2. Cell calcification model

The calcification of VSMCs was induced as described previously [28]. Briefly, P-VSMCs were cultured in DMEM, supplemented with 15% FBS, 10 mM β-GP, 50 mg/ml ascorbic acid, 10^{-7} M insulin, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin and neomycin for 8 d. For manipulation the activity of WNT/β-catenin signaling, WNT-3A (10 ng/ml) or DKK1 (100 ng/ml) was added without or with β-GP every 3 d.

2.3. Quantification of calcium deposition

P-VSMCs cultured in 10 mm dishes were washed twice with phosphate buffered saline and decalcified with 0.6 M HCl for 24 h. Calcium content of the supernatant was determined by the QuantiChrome Calcium Assay Kit (DICA-500, Bioassay Systems, Hayward, CA). After decalcification, cells were solubilized with a solution of 0.1 M NaOH and 0.1% sodium dodecylsulfate, and the protein content was measured by the BCA protein assay kit (Thermo Scientific, Rockford, IL). Calcium content of the cells was normalized to protein content and expressed as μg/mg protein.

2.4. Plasmid transient transfection

L-VSMCs seeded in six-well plates at 5×10^5 cells/well were transfected with Flag-tagged N-terminal truncated, stabilized β-catenin expression vector (pDel-β-cat) [29] for 48 h by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and the empty vector pcDNA3 (Invitrogen) was used as a mock transfection control. For some experiments, L-VSMCs were transient transfected with pcDNA3 or pDel-β-cat for 48 h, followed by treated with or without 10 mM β-GP for 24 h.

2.5. Reporter plasmid construction and site-directed mutagenesis

Runx2 promoter-luciferase reporters were constructed by cloning different lengths of the *Runx2* promoter region into the pGL3-Basic luciferase vector (Promega, Madison, WI). Different lengths of the rat *Runx2* gene promoter fragments were generated by using PCR and cloned into the pGL3-Basic luciferase vector using routine cloning procedures. The correct sequences of the *Runx2* promoter-luciferase constructs were confirmed by DNA sequencing at the Zoonbio Biotechnology (Nanjing, China) DNA sequencing core facility. For generating mutant *Runx2* promoter-luciferase reporter, either two points mutation (T to *C*, and G to *T*) at the TCF binding element (TBE) 1, or two points mutation (T to *C*, and G to *C*) at the TBE2, or all of the four points mutation were introduced in the 1.0kRunx2-Luc reporter plasmid by using site-

directed mutagenesis according to the protocol specified by the manufacturer (QuikChange II XL kit; Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing.

2.6. Luciferase assay

L-VSMCs were cotransfected by using Lipofectamine 2000 reagent with various *Runx2*-Luc luciferase reporter constructs (1.0 μg) plus active β-catenin expression vector β-cat(S37A) [30] or empty pcDNA3 vector (2.0 μg), respectively. A fixed amount (0.1 μg) of internal control reporter *Renilla reniformis* luciferase driven under a thymidine kinase promoter (pRL-TK; Promega) was also cotransfected for normalizing the transfection efficiency. In some experiments, the transfected cells were incubated with different concentration of β-GP or WNT-3A as indicated. Luciferase assay was carried out using a dual luciferase assay system kit according to the manufacturer's protocols (Promega). Relative luciferase activity was reported as fold induction over controls after normalizing for transfection efficiency.

2.7. Animal model

Male Sprague-Dawley rats weighted approximately 300–400 g were purchased from Shanghai Experimental Animal Center (Shanghai, China). They were housed in the animal facilities of the Experimental Animal Center of Nanjing Medical University with free access to food and water. Rat model of CRF with AMC was established according to the protocol described previously [6,31,32]. Briefly, male Sprague-Dawley rats were fed with a diet containing 0.75% adenine (Sigma-Aldrich) and 0.9% phosphorus. Rats fed with standard diet were used as normal controls. At 4 and 6 w after adenine-fed diet, groups of rats (n=4) were killed and aorta tissue and serum were collected for various analyses. Animals were treated humanely in accordance with the National Medical Advisory Committee Guidelines and the animal protocols were approved by the Institutional Animal Care and Use Committee at the Nanjing Medical University.

2.8. Alizarin red S staining and von Kossa staining

For determination of the calcium deposition in VSMCs, P-VSMCs after various treatment were incubated with 10% neutral buffered formalin for 10 min, followed by incubation with 2% aqueous alizarin red S solution (pH4.2) for 15 min.

For evaluation of the aortic calcification, paraffin-embedded artery sections (3 μm thickness) were prepared by a routine procedure. The sections were stained with 5% silver nitrate solution in a glass coplin jar placed under ultraviolet light for 1 h. Un-reacted silver was removed with 5% sodium thiosulfate for 5 min. Counter staining was performed in nuclear fast red for 2 min.

2.9. Western Blot analysis

The preparation of whole cell lysates and aorta tissue homogenates and Western blot analysis of protein expression were carried out by using established procedures as described previously [33]. The primary antibodies were obtained from the following sources: anti-Runx2 (ab76956; Abcam, Cambridge, MA), anti-dephosphorylated, active β-catenin (anti-ABC, clone 8E7, cat. no. 05-665; Merck Millipore, Billerica, MA), anti-phospho-β-catenin (Ser675) (#9567; Cell Signaling Technology, Beverly, MA), anti-β-catenin (cat. no. 610154; BD Transduction Laboratories, San Jose, CA), anti-Flag (M2, F3165; Sigma), anti-phospho-LRP6 (Ser1490) (#2568; Cell Signal), anti-LRP6 (C5C7) (#2560; Cell Signal), anti-β-actin (sc-1616; Santa Cruz Biotechnology, Dallas, TX), anti-TATA-binding protein (TBP) (ab181-100; Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778; Santa Cruz).

2.10. Real-time RT-PCR

Total RNA isolation and real-time RT-PCR were performed by the procedures described previously [34]. Briefly, the first strand cDNA synthesis was carried out by using a reverse transcription system kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time RT-PCR was performed on an ABI PRISM7300 sequence detection system (Applied Biosystems, Foster City, CA). The 25 μl volume of PCR reaction mixture contained 12.5 μl 2 × SYBR Green PCR Master Mix, 5 μl diluted (1:10) reverse transcriptase product, and 0.5 μM sense and antisense primer sets. The sequences of the primer pairs were as follows: Runx2, 5′-TGT GTG CCT CCA ACC TGT GT-3′ (sense) and 5′-CTT TCC CCC TCA ATT TGT GTC A-3′ (antisense); GAPDH, 5′-CAG CAA GGA TAC TGA GAG CAA GAG-3′ (sense) and 5′-GGA TGG AAT TGT GAG GGA GAT G-3′ (antisense). The Runx2 mRNA levels were calculated after normalizing with GAPDH.

2.11. Immunofluorescence staining

Immunofluorescence staining was carried out by using a routine procedure [34]. Briefly, cells cultured on coverslips were fixed with methanol and acetone (1:1) for 10 min at −20 °C. After blocking with 20% normal donkey serum for 30 min, cells were incubated with the specific primary antibodies against Runx2 and β-catenin, and then were stained with FITC- or cyanine Cy3-conjugated secondary antibody. Cells were double stained with 4′, 6-diamidino-2-phenylindole-HCl for visualizing the nuclei. Stained cells were mounted and viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri1, Nikon).

2.12. Immunohistochemical staining

Immunohistochemical staining of aorta sections was performed by an established protocol [28]. Paraffin-embedded sections were stained with anti-Runx2 or anti-β-catenin antibody using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA), according to the instructions of manufacturer. Slides were viewed under a Nikon Eclipse 80i microscope equipped with a digital camera (DS-Ri1, Nikon). The primary antibody was replaced by nonimmune normal control IgG as negative control, and no staining occurred.

2.13. Serum biochemistry

Serum concentrations of serum urea nitrogen, creatinine, inorganic phosphorus, and total calcium were measured by automated analyzer in the Center for Kidney Disease in the 2nd Affiliated Hospital of Nanjing Medical University.

2.14. Statistical analysis

Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups were made using one-way analysis of variance, followed by the Student-Newman-Keul's test. A p value of <0.05 was considered significant.

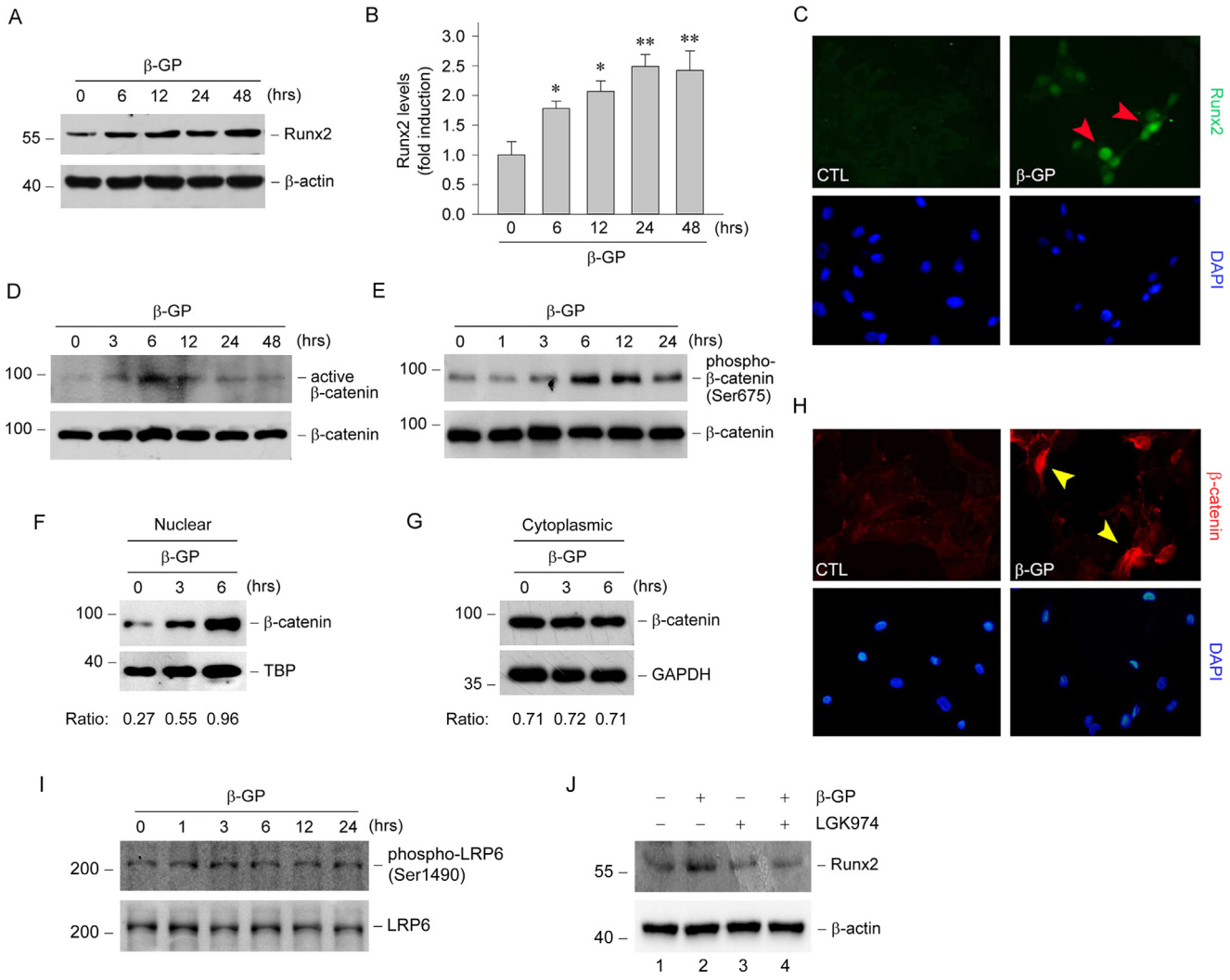

Fig. 1. β-GP stimulates Runx2 induction and activates β-catenin signaling in VSMCs. (A-C) β-GP induces Runx2 expression in VSMCs. VSMCs were treated with 10 mM β-GP for various periods of time as indicated, and the cell lysates were immunoblotted with antibodies against Runx2 and β-actin, respectively. A representative Western blot (A) and the quantitative data (B) are shown. *, $p < 0.05$, **, $p < 0.01$ *versus* without β-GP ($n=3$). Immunofluorescence staining shows Runx2 expression and localization in VSMCs incubated with 10 mM β-GP for 24 h. Arrowheads indicate positive nuclear staining of Runx2 (C). (D, E) β-GP induces β-catenin activation in VSMCs. VSMCs were treated with 10 mM β-GP for various periods of time as indicated, and the cell lysates were immunoblotted with antibodies against active β-catenin (D), phospho-β-catenin (Ser675) (E), and total β-catenin (D, E), respectively. (F-H) High-phosphate induces β-catenin nuclear translocation in VSMCs. VSMCs were treated with 10 mM β-GP for 3 or 6 h, and nuclear and cytoplasmic protein from VSMCs were prepared and immunoblotted with antibodies against β-catenin, TBP or GAPDH, respectively. The ratios of β-catenin per control proteins (TBP for nuclear protein and GAPDH for cytoplasmic protein) are shown (F, G). Immunofluorescence staining shows β-catenin expression in VSMCs treated with 10 mM β-GP for 6 h. Arrowheads denote nuclear localization of β-catenin (H). (I) β-GP induces LRP6 phosphorylation. VSMCs were treated with 10 mM β-GP for various periods of time as indicated, and the cell lysates were immunoblotted with antibodies against phospho-LRP6 (Ser1490) and LRP6 (C5C7), respectively. (J) Inhibition of PORCN abolishes Runx2 induction stimulated by β-GP. VSMCs were pretreated with LGK 974 (1 nM) for 30 min, then incubated with β-GP (10 mM) for 24 h, and the cell lysates were immunoblotted with antibodies against Runx2 and β-actin, respectively. β-GP, β-glycerophosphoric acid; CTL, control; DAPI, 4′, 6-diamidino-2-phenylindole-HCl; TBP, TATA-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LRP, low density lipoprotein receptor-related protein.

3. Results

3.1. β-GP induces Runx2 expression and WNT/β-catenin signaling activation in VSMCs

Induction of osteogenic genes, such as *Runx2*, is one of the main features of VOT in a high-phosphate environment. We first examined the expression of Runx2 by incubating VSMCs with β-GP, a donor of organic phosphate [28]. As shown in Fig. 1A, β-GP markedly induced Runx2 expression in a time-dependent manner. Quantitative determination revealed a 2.5-fold induction of Runx2 protein at 24 h after β-GP (10.0 mM) treatment in VSMCs (Fig. 1B). Immunofluorescence staining exhibited a notable, nuclear localization of Runx2 in VSMCs stimulated by β-GP (Fig. 1C, arrowheads), whereas the staining for Runx2 at basal condition was weak and misty in cytoplasm (Fig. 1C).

Several studies have shown that WNT/β-catenin signaling is involved in the pathogenesis of VOT [23–26], which motivated us to explore whether this signal pathway plays a role in mediating β-GP-induced Runx2 expression in VSMCs. For this purpose, we investigated the regulation of β-catenin activity by β-GP. As shown in Fig. 1D and E, β-GP stimulated β-catenin activation in VSMCs, as revealed by the upregulation of both dephosphorylated (Ser37/Thr41) β-catenin (Fig. 1D) and phosphorylated (Ser675) β-catenin (Fig. 1E). Given that either dephosphorylation on Ser37/Thr41 sites or phosphorylation on Ser675 site induces β-catenin translocation into the nuclei and increases its transcriptional activity [17,18], we next tested whether β-GP induced β-catenin nuclear translocation, an obligatory step for β-catenin to govern its target gene transcription. As shown in Fig. 1F, treatment with β-GP promoted β-catenin nuclear translocation in VSMCs. Of note, although β-GP caused a predominant increase of β-catenin in nuclei, the

abundance of β-catenin in cytoplasm seemed almost no change (Fig. 1G), and the total β-catenin levels were little affected (Fig. 1D and E). Immunofluorescence staining exhibited nuclear localization of β-catenin in VSMCs incubated with β-GP (10.0 mM) for 6 h (Fig. 1H, arrowheads), whereas β-catenin located in cell-cell junction and cytoplasm under basal circumstance (Fig. 1H). Hence, high-phosphate induced Runx2 expression along with activation of β-catenin in VSMCs.

In order to confirm WNT/β-catenin signaling involving in the induction of Runx2 by high-phosphate, we tested two crucial steps in upstream of this signaling. One is phosphorylation of LRP6, another is secretion of WNT. As one of coreceptors for WNT, LRP6 is required for WNT/β-catenin signaling. Upon stimulation with WNT, LRP6 is phosphorylated at multiple sites including Thr1479, Ser1490 and Thr1493 [35,36]. As shown in Fig. 1I, it seemed that the phosphorylation of LRP6 (Ser1490) was induced within 3 h after β-GP treatment in VSMCs. This result suggests that high-phosphate may evoke the enhancement of autocrine WNT because LRP6 phosphorylation is WNT-dependent, so we next assessed the necessity of WNT secretion for high-phosphate-induced Runx2 expression. To this end, VSMCs were treated with LGK974, a potent and specific small molecule PORCN inhibitor, followed by incubation with β-GP. As an o-acyltransferase, PORCN is required for palmitoylation of WNT ligands, an indispensable step in the processing of WNT ligand secretion [37]. As shown in Fig. 1J, inhibition of PORCN abolished Runx2 induction by β-GP in VSMCs (Fig. 1J, lane4 *versus* lane2). Considered together, high-phosphate appears to activate WNT/β-catenin signaling in VSMCs via promoting WNT secretion.

3.2. Activation of β-catenin signaling mediates Runx2 induction in VSMCs

To determine any involvement of β-catenin activation in Runx2 regulation, VSMCs were transiently transfected with Flag-tagged, N-terminal truncated, stabilized β-catenin expression vector (pDel-β-cat) [29] or empty vector (pcDNA3). As shown in Fig. 2A, elevated level of exogenous stabilized β-catenin in the VSMCs transfected with pDel-β-cat was evident, as presented by Western blot analysis using anti-Flag antibody, and ectopic expression of stabilized β-catenin resulted in Runx2 induction. To address whether Runx2 induction by β-catenin activation is a general phenomenon, we also examined Runx2 expression in the VSMCs transiently transfected with another stabilized β-catenin expression vector (β-cat(S37A)) [30] and got a similar result (data not shown). Quantitative determination revealed an approximate three-fold induction of Runx2 expression in the VSMCs transfected with pDel-β-cat, compared with that transfected with pcDNA3 (Fig. 2B). Immunofluorescence staining presented a remarkable nuclear localization of Runx2 in the VSMCs overexpressing stabilized β-catenin (Fig. 2C, arrows). However, it seemed that exogenous stabilized β-catenin couldn't further promote Runx2 induction at β-GP-stimulated conditions (Fig. 2D, lane4 *versus* lane2 and lane3, respectively, and Fig. 2E).

Given that the inhibition of the β-catenin degradation complex can be achieved by direct inhibition of GSK-3β, we assessed the effect of IM-12, a selective small molecule GSK-3β inhibitor [38], on Runx2 induction in VSMCs. We found that activation of β-catenin signaling through the inhibition of GSK-3β also induced the

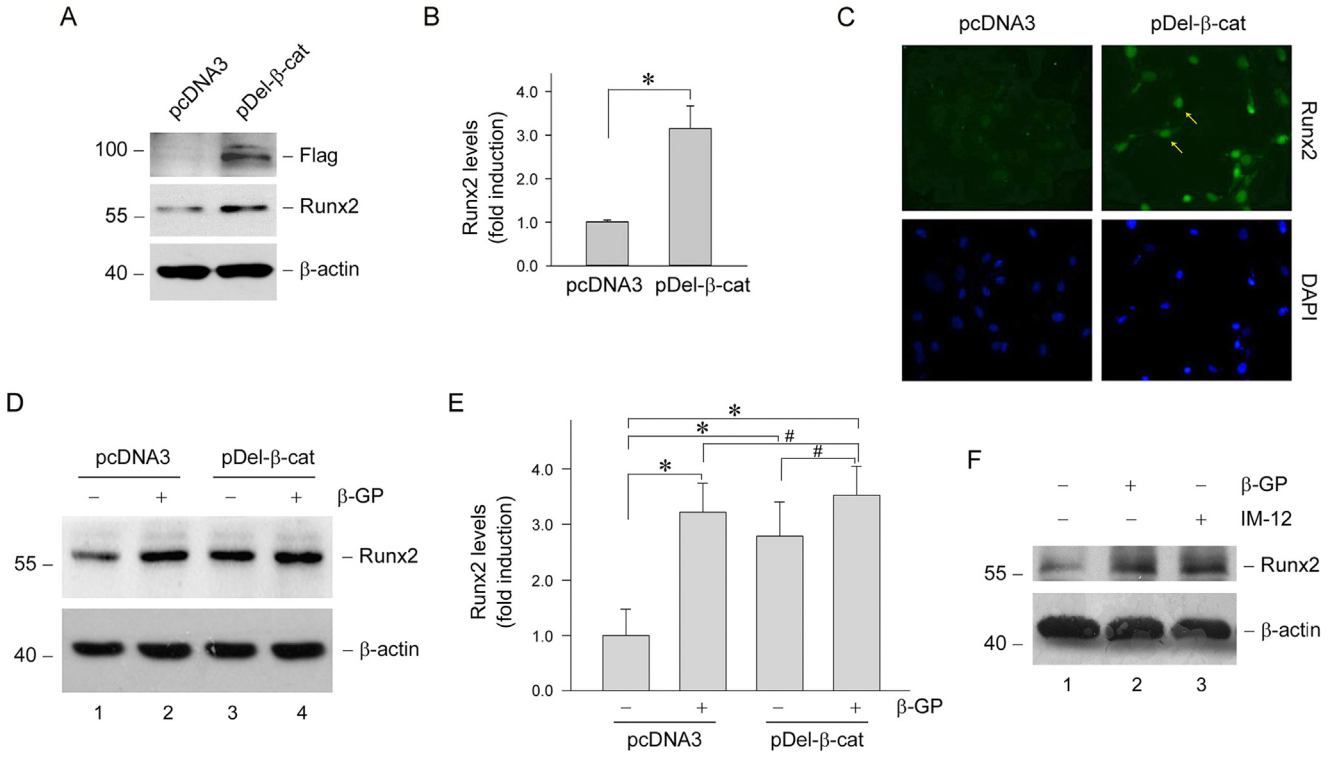

Fig. 2. Activation of β-catenin signaling promotes Runx2 induction in VSMCs. (A-C) Ectopic expression of stabilized β-catenin promotes Runx2 induction. L-VSMCs were transiently transfected with either empty vector (pcDNA3) or Flag-tagged, N-terminal truncated β-catenin vector (pDel-β-cat) for 48 h, and the cell lysates were immunoblotted with antibodies against Flag, Runx2, and β-actin, respectively. A representative Western blot (A) and the quantitative data (B) are presented. *, $p < 0.05$ *versus* pcDNA3 controls ($n=3$). Immunofluorescence staining shows Runx2 expression in VSMCs transfected with either pcDNA3 or pDel-β-cat for 48 h. Arrows indicate positive nuclear staining of Runx2 in VSMCs (C). (D, E) Exogenous β-catenin couldn't further promote Runx2 induction at β-GP-stimulated conditions. L-VSMCs were transiently transfected with either pcDNA3 or pDel-β-cat for 24 h, and followed by treatment with or without 10 mM β-GP for another 24 h. A representative Western blot (D) and the quantitative data (E) are shown. *, $p < 0.05$ *versus* pcDNA3 without β-GP. #, $p > 0.05$ *versus* pDel-β-cat with β-GP ($n=3$). (F) Inhibition of GSK-3β promotes Runx2 induction. VSMCs were treated with or without IM-12 (3 μM) for 24 h, and cells treated with β-GP (10 mM) were as a positive control. The cell lysates were immunoblotted with antibodies against Runx2 and β-actin, respectively. β-GP, β-glycerophosphoric acid; DAPI, 4′, 6-diamidino-2-phenylindole-HCl.

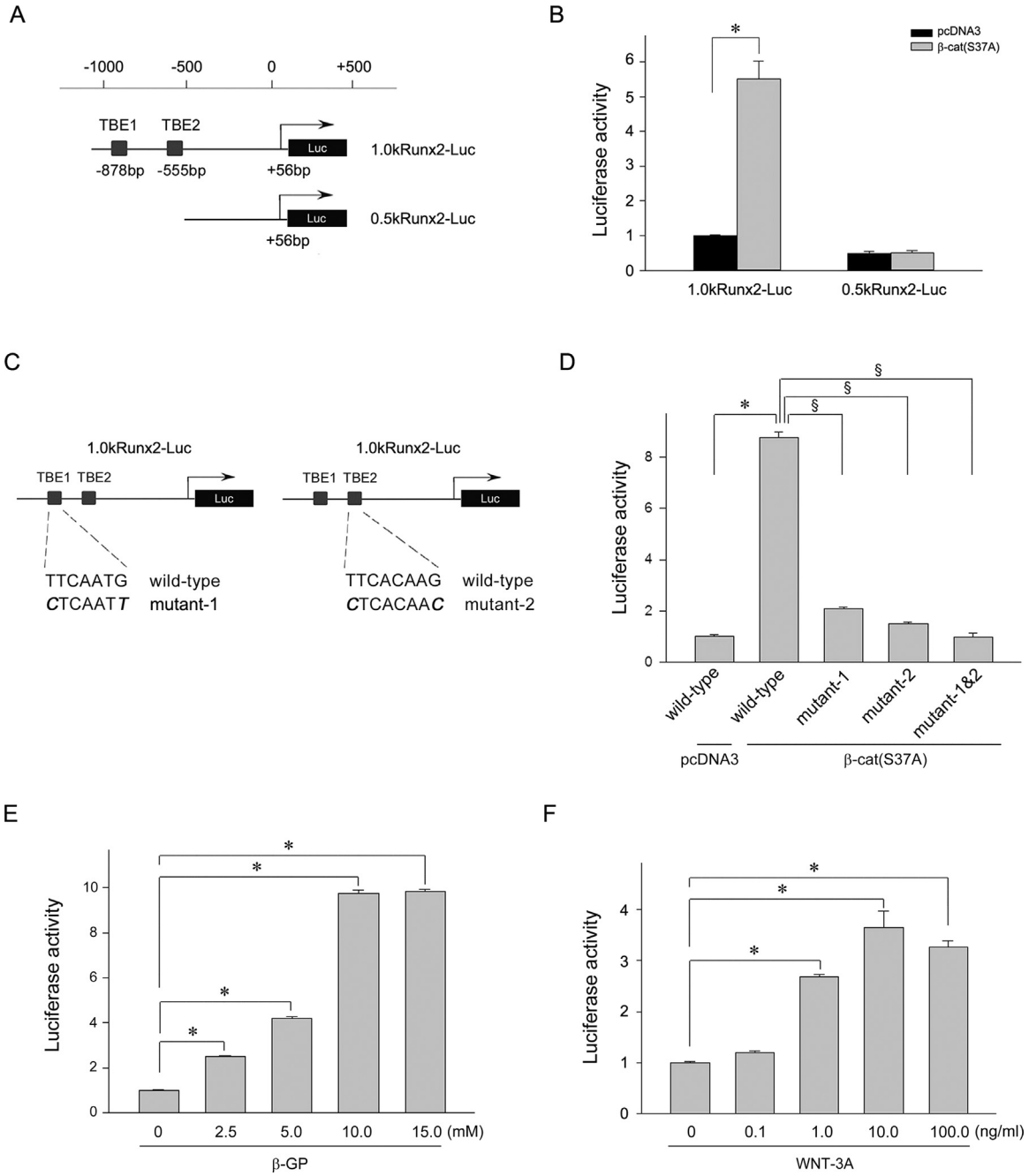

Fig. 3. Runx2 gene promoter contains two functional TBEs which are responsible for β-catenin-mediated Runx2 induction in VSMCs. (A) Diagram shows the construction of the luciferase reporter plasmid containing the promoter region of rat *Runx2* gene linked to the coding sequence of luciferase. Putative two TBE sites are indicated. (B) L-VSMCs were cotransfected with the luciferase reporter plasmid (1.0kRunx2-Luc or 0.5kRunx2-Luc) and either empty vector (pcDNA3) or stabilized β-catenin vector (β-cat (S37A)) for 48 h. Luciferase activities show that *Runx2* promoter contains at least one functional TBE (1.0kRunx2-Luc) that is responsive to β-catenin stimulation. (C, D) site-directed mutation in either (mutant-1 in TBE1 or mutant-2 in TBE2) or both (mutant-1 and 2) of two TBEs of *Runx2* promoter abolishes its responsiveness to β-catenin stimulation. The construction of wild-type and mutant TBE reporter plasmids (C) and luciferase activities (D) are presented. *, $p < 0.01$ *versus* pcDNA3 controls; §, $p < 0.01$ *versus* cotransfected with β-cat(S37A) and wile type luciferase reporter plasmid (1.0kRunx2-Luc) ($n=3$). (E, F) High-phosphate or WNT-3A enhances Runx2 luciferase reporter activity. L-VSMCs were transfected with the luciferase reporter plasmid (1.0kRunx2-Luc). Twenty-four hours after transfection, the cells were incubated with various concentrations of β-GP (E) or WNT-3A protein (F) as indicated. *, $p < 0.01$ *versus* vehicle controls ($n=3$).

expression of Runx2 (Fig. 2F, lane3 *versus* lane1).

3.3. *Runx2 gene promoter contains two functional TCF binding sites responsible for β-catenin action*

To elucidate the mechanism underlying β-catenin regulation of Runx2 expression in VSMCs, we analyzed the structure and function of rat *Runx2* gene promoter. Bioinformatics analysis revealed two putative TCF-binding sites (TBE1 and TBE2) in the proximal promoter region of the rat *Runx2* gene (Fig. 3A). To test the functionality of these TBEs, we constructed two luciferase reporter plasmids containing different lengths of the promoter region of the rat *Runx2* gene linked to the coding sequence of luciferase gene. After these constructs were cotransfected into VSMCs with empty vector pcDNA3 or stabilized β-catenin expression vector β-cat(S37A) [30], luciferase activities were assessed. As shown in Fig. 3B, at least one functional TBE, located at −1000bp to −500bp relative to transcription initiation site, was responsive to the

stimulation from β-catenin. Deletion of this region abolished luciferase induction by β-catenin (Fig. 3B). To confirm the importance of these TBEs in regulating Runx2 expression, we utilized a site-directed mutagenesis approach to introduce two points mutation in the TBE1 (T to *C*, and G to *T*) and TBE2 (T to *C*, and G to *C*), respectively (Fig. 3C). As shown in Fig. 3D, damage of each of two TBEs largely attenuated its susceptibility to β-catenin stimulation, while destruction both of them by introducing all of these four points mutation almost completely abolished its responsiveness to β-catenin.

Next, we tested the effect of high-phosphate on Runx2 promoter activity. We found that β-GP stimulated Runx2 promoter activity in a dose-dependent manner in VSMCs after transfection with Runx2 promoter-luciferase reporter plasmid (1.0kRunx2-Luc) (Fig. 3E). This result is consistent with the induction of Runx2 protein by β-GP (Fig. 1A), suggesting that high-phosphate, through activation of WNT/β-catenin signaling, is able to activate Runx2 promoter and induce its gene expression. Since WNT is the upstream activator of β-catenin signaling, we examined the effect of WNT-3A, the prototypic WNT that transmits its signal via β-catenin-dependent signaling, on Runx2 promoter activity in VSMCs. As shown in Fig. 3F, the activation of β-catenin signaling by WNT-3A also induced Runx2 promoter activity in VSMCs.

3.4. WNT/β-catenin signaling modulates VSMCs osteogenic transdifferentiation and calcification in vitro

To provide further evidence for a role of β-catenin-dependent

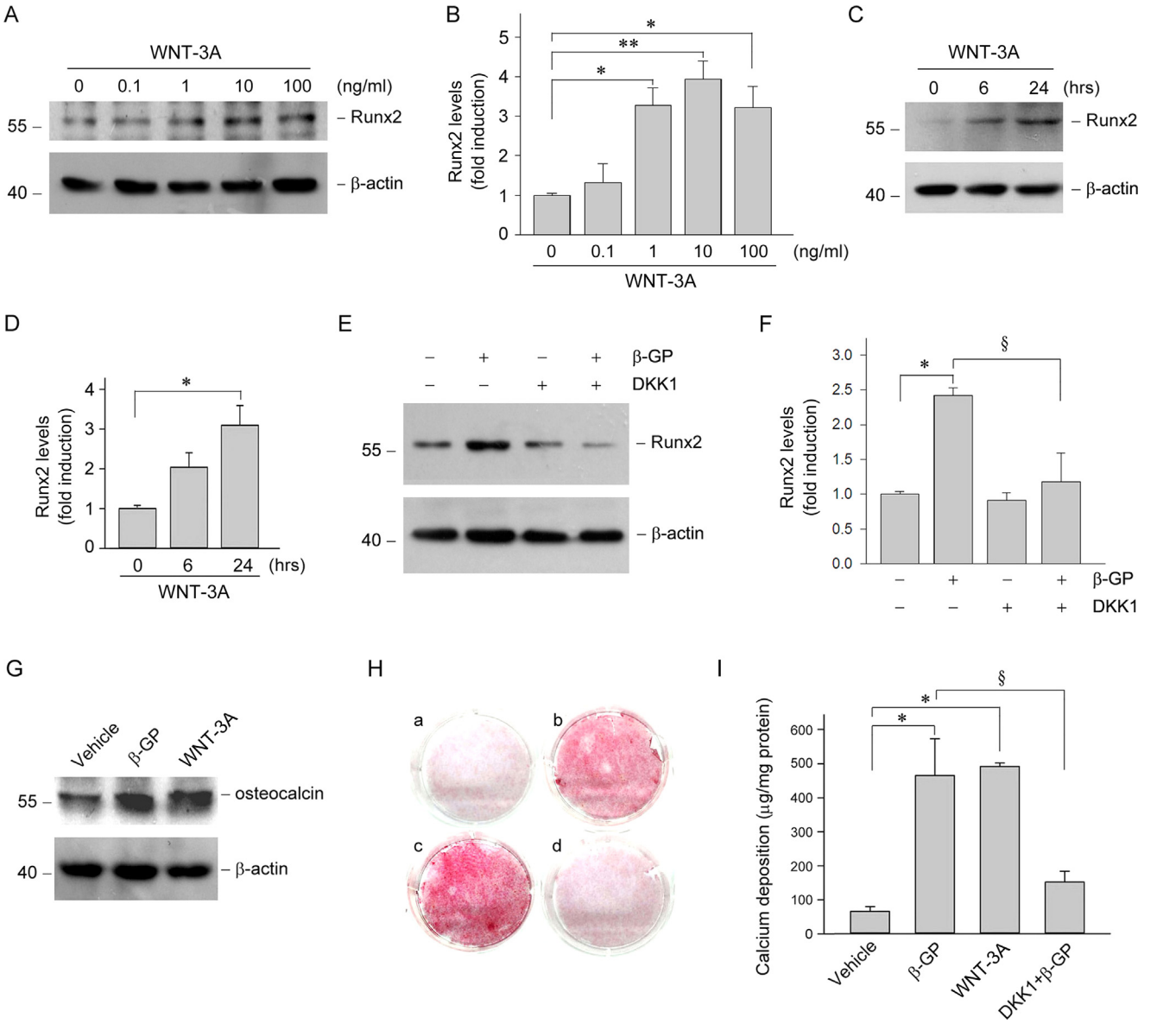

Fig. 4. WNT/β-catenin signaling modulates VSMCs osteogenic transdifferentiation and calcification *in vitro*. (A-D) WNT-3A promotes Runx2 expression. VSMCs were treated with different concentrations of WNT-3A for 24 h or 10 ng/ml WNT-3A for various periods of time as indicated. Representative Western blots (A, C) and the quantitative data (B, D) are shown. *, $p < 0.05$,**, $p < 0.01$ *versus* without WNT-3A ($n=3$). (E, F), DKK1 inhibits Runx2 expression induced by β-GP. VSMCs were pretreated with DKK1 (100 ng/ml) for 30 min, then incubated with β-GP (10 mM) for 24 h. A representative Western blot (E) and the quantitative data (F) are presented. *, $p < 0.01$ *versus* without β-GP; §, $p < 0.01$ *versus* without DKK1 ($n=3$). (G) Activation of WNT/β-catenin signaling by either β-GP or WNT-3A upregulates osteocalcin expression in VSMCs. VSMCs were treated with 10 mM β-GP or 10 ng/ml WNT-3A for 6 d, and the cell lysates were immunoblotted with antibodies against osteocalcin and β-actin, respectively. (H, I) WNT/β-catenin signaling modulates VSMCs calcification. VSMCs were treated with various stimulators for 8 d. Alizarin red S staining (H) and the results of calcium content measured by a quantitative method (I) in VSMCs are presented. a, Vehicle; b, 10 mM β-GP; c, 10 ng/ml WNT-3A; and d, 10 mM β-GP with 100 ng/ml DKK1. *, $p < 0.05$ *versus* vehicle controls; §, $p < 0.05$ *versus* β-GP alone ($n=3$).

signaling in regulating Runx2 expression, we next examined the effect of WNT-3A on Runx2 expression in VSMCs. As shown in Fig. 4A and C, Runx2 was induced in VSMCs treated with recombinant WNT-3A protein in a dose- or time-dependent manner. Quantitative determination displayed a three- to four-fold induction of Runx2 protein at 24 h after WNT-3A (10 ng/ml) treatment in VSMCs (Fig. 4B and D). For assessing the necessity of WNT/β-catenin signaling activation for β-GP-induced Runx2 expression, we further investigated the inhibitory effect of WNT antagonist DKK1, a natural inhibitor of WNT/β-catenin signaling by the specialty to interact with and block LRP5/6 coreceptor, on the induction of Runx2 in VSMCs stimulated by β-GP. For this purpose, VSMCs were treated with recombinant DKK1 protein (100 ng/ml), followed by incubation with β-GP. As shown in Fig. 4E and F, inhibition of WNT/β-catenin signaling attenuated Runx2 induction by β-GP in VSMCs.

We next observed the influence of WNT/β-catenin signaling activation on the expression of osteocalcin, a bone-related protein which is downstream of Runx2, in VSMCs. As shown in Fig. 4G, WNT-3A promoted osteocalcin induction in VSMCs, similar to the effect of β-GP. We further investigated the effect of WNT/β-catenin signaling on calcification in VSMCs. As displayed in Fig. 4H, activation of WNT/β-catenin signaling by WNT-3A induced calcium deposition in VSMCs (Fig. 4H, c *versus* a), similar to the effect produced by β-GP (Fig. 4H, c *versus* b), whereas inhibition of WNT/β-catenin signaling by DKK1 markedly abolished calcium deposition stimulated by β-GP (Fig. 4H, d *versus* b). By using a quantitative method, we examined the calcium contents in VSMCs treated with various stimulators and found the similar results (Fig. 4I). Therefore, it is suggested that WNT-3A, through activation of its downstream mediator β-catenin, is able to induce Runx2 and osteocalcin expression and promote calcification in VSMCs. Conversely, blockade of WNT/β-catenin signaling by DKK1 inhibits Runx2 induction and calcium deposition stimulated by β-GP.

3.5. *Runx2 expression is positively associated with β-catenin activation in the aortic wall of CRF rats*

To investigate the potential relationship of activity of WNT/β-catenin signaling to Runx2 expression *in vivo*, we established CRF with AMC model in rats. The adenine-fed rats had severe renal failure, with a remarkable increase in serum creatinine (5.37 ± 1.29 mg/dl and 4.64 ± 0.23 mg/dl in 4 and 6 w adenine-fed rats *versus* 1.66 ± 0.23 mg/dl in controls, respectively) and serum urea nitrogen (132.46 ± 28.34 mg/dl and 169.99 ± 16.63 mg/dl, *versus* 24.53 ± 1.13 mg/dl, respectively) (Fig. 5E). The 4 and 6 w adenine-fed rats showed an evident increase in serum phosphorus (5.92 ± 0.74 mmol/l and 6.30 ± 0.78 mmol/l) compared with controls (2.45 ± 0.02 mmol/l) (Fig. 5E), similar to a previous report [32]. Histological assessment using von Kossa staining displayed that the aortic wall in 6 w adenine-fed rats had extensive linear calcification, which was localized in the aortic media, resembling the typical Mönckeberg's pattern (Fig. 5B *versus* A, and D *versus* C).

Then, we examined Runx2 and β-catenin protein expression and localization in the aortic wall of 4 w CRF rats. Immunohistochemical staining showed that Runx2 expression was mainly induced in the aortic media of CRF rats compared with controls (Fig. 6B *versus* A). Meanwhile, the cell numbers of positive β-catenin staining were markedly increased in the aortic wall of CRF rats compared with controls (Fig. 6D *versus* C, and F *versus* E), and β-catenin was predominantly localized in the nuclei of VSMCs in the media of aorta (Fig. 6F, arrowheads). Consistently, Runx2 mRNA expression was remarkable upregulation in the aortic wall of 4 and 6 w CRF rats, as compared with the normal controls, which was determined by quantitative real-time RT-PCR approach (Fig. 6G). Next, we investigated the expression of dephosphorylated β-catenin (Ser37/Thr41) and phosphorylated β-catenin (Ser675), two active forms of β-catenin, in the aortic wall of various groups. Western blot analysis showed a dramatic increase in both of them despite exhibiting in different expression patterns (Fig. 6H and I). Quantitative determination indicated approximately six-fold induction of the relative abundance of phosphorylated β-catenin (Ser675) protein in the aortic wall of 4 w CRF rats, when compared with that in the normal controls (Fig. 6J). Linear regression showed a positive correlation between Runx2 mRNA level and phosphorylated β-catenin (Ser675) abundance (Fig. 6K). These observations suggest that β-catenin activation may implicate in Runx2 induction and osteogenic transdifferentiation

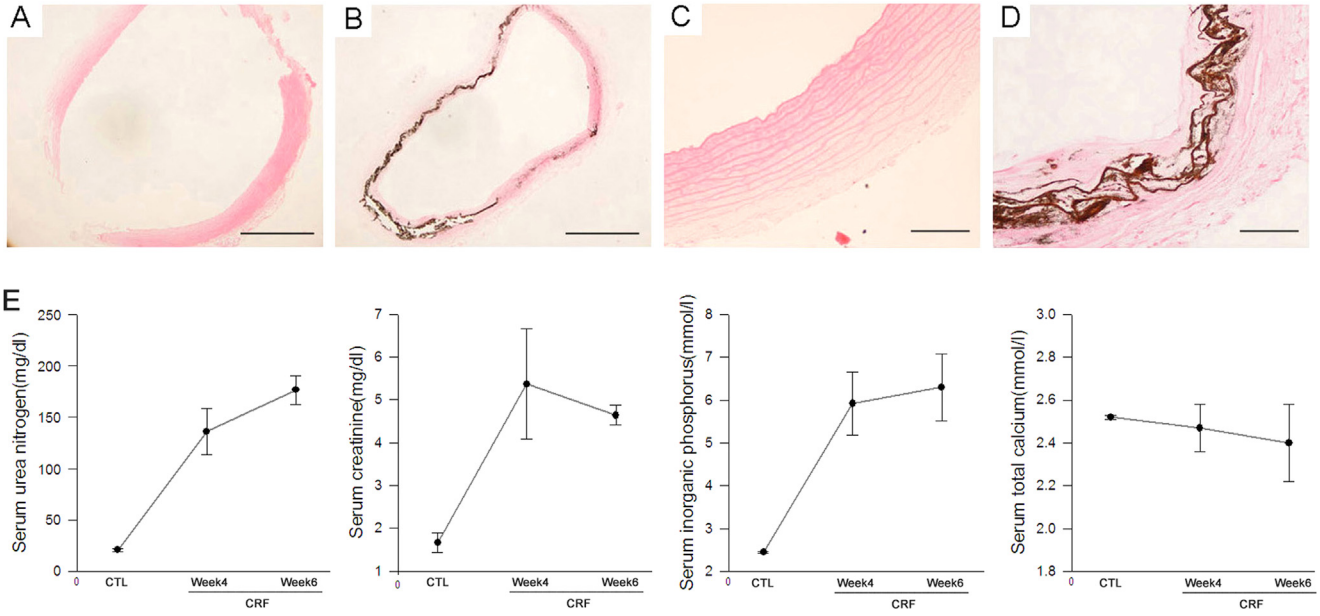

Fig. 5. Arterial medial calcification is induced in rats with chronic renal failure. (A-D) Morphological assessment presents the extensive calcium deposition in the tunica media of the aorta in rats with chronic renal failure (CRF). Aortic section from the normal controls (A, C) and adenine-fed 6 w CRF rats (B, D) were processed with von Kossa staining. Scale bar, 500 μm (A, B); 50 μm (C, D). (E) The quantitative data of serum urea nitrogen, creatinine, inorganic phosphorus and total calcium in each group are shown. ($n=4$).

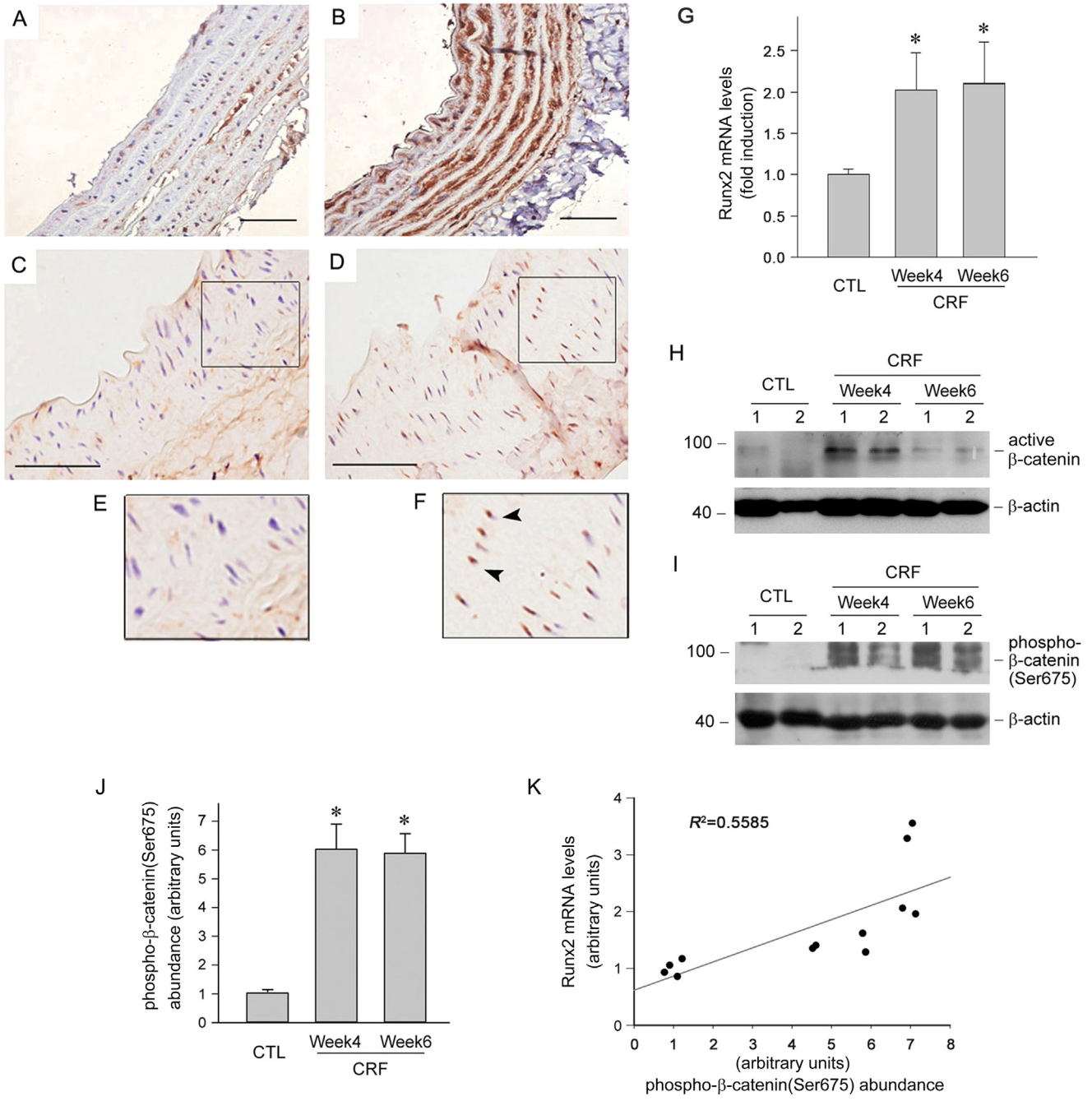

Fig. 6. Runx2 expression is positively correlated with β-catenin activation in the aortic wall of rats with chronic renal failure. (A-F) Immunohistochemical staining shows the expression and localization of Runx2 (A, B) and β-catenin (C-F) protein in the aortic wall of control rats and CRF rats, respectively. Aortic sections from normal controls (A, C) and adenine-fed 4 w CRF rats (B, D) were immunostained with antibodies against Runx2 and β-catenin, respectively. Scale bar, 50 μm (A, B); 100 μm (C, D). Enlarged image from the boxed area in C and D are shown (E, F) and the arrowheads indicate β-catenin positive cells (F). (G) Quantitative, real-time RT-PCR analyses show the mRNA levels of Runx2 in each group. *, $p < 0.05$ *versus* controls (CTL) ($n=4$). (H, I) β-catenin is activated in the aortic wall of rats with CRF. The aortic tissue lysates from each group were immunoblotted with antibodies against active β-catenin (H), phospho-β-catenin (Ser675) (I) and β-actin, respectively. (J) The quantitative data of Western blot analysis for phospho-β-catenin (Ser675) are presented. *, $p < 0.05$ *versus* controls ($n=4$). (K) Linear regression shows a positively correlation between aortic Runx2 mRNA levels and phospho-β-catenin(Ser675) abundance (arbitrary units). The correlation coefficient (R^2) is shown.

in the VSMCs of aortic media in CRF rats during the process of AMC.

4. Discussion

It has been demonstrated that WNT/β-catenin signaling promotes osteogenesis by directly stimulating Runx2 gene expression [12]. However, the cell-specific transcriptional mechanism through which this signaling regulates Runx2 expression in VSMCs remains incompletely understood. Our findings in present study suggest that Runx2 is a transcriptional target of WNT/β-catenin signaling pathway in the VSMCs undergoing VOT in a high-phosphate environment. Evidence suggestive of this conclusion is as follows. First, Runx2 expression is induced and β-catenin is activated by high-phosphate, and activation of β-catenin signaling by overexpressing stabilized β-catenin or inhibiting GSK-3β induces Runx2 expression in VSMCs. Second, the Runx2 gene promoter region contains two TBEs, which functionally mediate the interaction with TCF in response to β-catenin activation. Third,

activation of β-catenin by WNT-3A induces Runx2 expression, whereas inhibition of WNT/β-catenin signaling by PORCN inhibitor or DKK1 attenuates Runx2 induction stimulated by high-phosphate in VSMCs. Fourth, WNT-3A upregulates osteocalcin expression and promotes calcium deposition in VSMCs, whereas DKK1 inhibits VSMCs calcification induced by high-phosphate. Finally, in CRF rats, β-catenin is activated and Runx2 is induced in the aortic tunica media, followed by AMC, and the Runx2 mRNA level is positively correlated with the abundance of active β-catenin in the aortic wall. Given that high-phosphate activates β-catenin in VSMCs, this signal pathway likely plays a critical role in mediating high-phosphate-triggered Runx2 induction. Our studies provide significant insights into the role and mechanism of WNT/β-catenin signaling in regulation of Runx2 expression during VOT. Furthermore, these results suggest that Runx2 induction could play an essential role in mediating the action of WNT/β-catenin signaling in promoting vascular calcification.

Vascular calcification has been recognized to occur as a consequence of the processes similar to bone formation [2,3]. As a cell type derived from mesenchymal cell, VSMCs under stress can differentiate to other mesenchymal origin cell types, such as osteoblasts. At the site of calcification, VSMCs undergo a phenotypic change to osteoblast-like cells with upregulating the expression of several bone-related proteins, which are normally confined to regulate the procedure of mineralization in bone [39]. These proteins include a number of transcription factors, such as Runx2, Osterix, Msx2, and Sox9, which are involved in the differentiation of VSMCs to an osteoblastic phenotype [39]. As previously demonstrated by us [28] and other researchers [24,25,27,40–42], VSMCs cultured in high-phosphate undergo VSMCs to osteogenic transdifferentiation (VOT) and calcification. As a master osteoblast transcription factor and the earliest osteoblastic marker, Runx2 has been generally recognized as an early feature of VOT [24,25,27,28]. This notion is further strengthened by present study exhibiting that Runx2 is markedly induced in the VSMCs in a high-phosphate environment both *in vitro* and *in vivo* (Fig. 1A through C, 6B, G and H), especially in the nuclei of the VSMCs (Fig. 1C, arrowheads).

WNT/β-catenin signaling has been implicated in the regulation of high-phosphate-induced VOT and calcification of VSMCs [23–27,43]. Several studies indicate that high-phosphate can activate β-catenin signaling, which is evidenced by the upregulation of β-catenin expression [25,26], the decrease of phosphorylated β-catenin (Ser33/37/Thr41) [26], or the translocation of β-catenin into the nuclei [24,27] in VSMCs. β-Catenin specifically plays a two-tier role in cell function. On the one hand, it is an indispensable structural component of adherens junctions based on cadherin; on the other hand, it is the pivotal effector of WNT/β-catenin signaling in the nucleus [35]. If not combined with cadherins, cytoplasmic β-catenin is phosphorylated by a multiprotein destruction complex which is comprised of the scaffold proteins Axin and adenomatous polyposis coli, casein kinase 1α (CK1α), and glycogen synthase kinase 3 (GSK3). β-Catenin is phosphorylated on Ser45 site by CK1α and then on Ser33/Ser37/Thr41 sites by GSK3, making its ubiquitination and proteasomal degradation [35]. So without a WNT signal, the levels of cytoplasmic free β-catenin are maintained low. WNT signaling causes the disintegration of the destruction complex and the inhibition of GSK3 activity, leading to a reduced phosphorylation of β-catenin on Ser33/Ser37/Thr41 sites, which results in its stabilization, accumulation and subsequent translocation into the nucleus [35]. In addition to this WNT/β-catenin signaling pathway, phosphorylation of β-catenin on Ser675 site by either protein kinase A (PKA) [17,18] or p21-activated kinase 1 (PAK1) [19] promotes TCF-dependent gene transcription, thereby enhancing the signaling activity of β-catenin. These studies indicated that phosphorylation of β-catenin on Ser675 site promotes the stability of β-catenin [18,19], or increases the recruitment of coactivators of β-catenin-mediated transcription, such as CREB binding protein [17]. In present study, it is demonstrated that high-phosphate activates WNT/β-catenin signaling in VSMCs, which is confirmed by the translocation of β-catenin into the nuclei. In addition, what is more interesting is that our finding indicates that high-phosphate induces two forms of active β-catenin both *in vitro* and *in vivo*: one is the β-catenin dephosphorylated on Ser37/Thr41 sites (Figs. 1D and 6H), another is the β-catenin phosphorylated on Ser675 site (Figs. 1E, and 6I). This finding suggests that high-phosphate may activate WNT/β-catenin signaling in VSMCs via at least two pathways, which lead to the inhibition of GSK3 and the activation of PKA or/and PAK1, respectively, resulting in the enhancement of stability and transcriptional activity of β-catenin. However, the potential mechanism that how high-phosphate modulates the activity of these kinases in VSMCs still need be clarified by further studies.

The activity of WNT/β-catenin signaling is dependent on WNT ligand. In the process of WNT ligand biosynthesis, posttranslational palmitoylation of WNT is mediated by PORCN, which is required for subsequent WNT secretion. Our finding suggests that high-phosphate may activate WNT/β-catenin signaling via enhancing the activity of PORCN in VSMCs and promoting WNT ligand secretion, which in turn induces the phosphorylation of LRP6. Likewise, the mechanism of high-phosphate regulating PORCN in VSMCs also need be cleared by further studies.

One major finding of present study is the identification of Runx2 as a directly transcriptional target of WNT/β-catenin signaling in VSMCs. Previous study demonstrates that WNT/β-catenin signaling positively regulates *Runx2* promoter activity in mouse osteoprogenitor (MC3T3) cells and embryonic mesenchymal cells prior to the induction of osteoblast phenotypic genes, and a functional TCF regulatory element responsive to WNT/β-catenin signaling is determined to reside in the promoter of the *Runx2* gene in MC3T3 cells [12]. In present study, activation of β-catenin signaling by overexpression of stabilized, dephosphorylated β-catenin (β-cat(S37A)) induces *Runx2* promoter activity in VSMCs. Our finding identifies two critical TBEs located at −1000bp to −500bp relative to transcription initiation site, which are responsive to exogenous β-catenin stimulation in VSMCs (Fig. 3A). Deletion or destruction of both of the TBEs completely abolishes its responsiveness to β-catenin stimulation (Fig. 3B and D). Of note, destruction of either of the TBEs is able to largely attenuate its responsiveness to β-catenin signal, suggesting that the functional response of two TBEs to β-catenin signal seems interactive and synergistic. To our knowledge, this is the first report which is unveiling the role of WNT/β-catenin signaling in regulating *Runx2* gene expression in VSMCs.

WNT-3A is an upstream activator in WNT/β-catenin signaling, while β-GP also has the ability to activate this signaling in VSMCs according to our findings in present study (Fig. 1), so it is not surprising that both factors can stimulate Runx2 promoter activity (Fig. 3E and F). Given that WNT/β-catenin signaling is inactive in VSMCs under normal conditions, the high-phosphate-induced signaling activation suggests that the signaling machinery is existent in the cells. Previous study demonstrates that several WNT ligands (*e.g.* WNT-2B, WNT-10B, and WNT-7A) and other WNT/β-catenin signaling-related genes, such as receptors, co-receptors, antagonists, inhibitors, and so on, are expressed detectably in normal VSMCs [44]. We hypothesize that high-phosphate may break the balance among these signaling components through some mechanism, for example, enhancing WNT secretion. Since multiple ligands and receptors could create combinatorial diversity and contribute to variable cellular responses [45], the activation of signaling induced by high-phosphate is a comprehensive effect. Therefore, it could be possible that β-GP induces a stronger Runx2 promoter activity than WNT-3A does (Fig. 3E

versus F).

Our study also suggests that targeting WNT/β-catenin signaling might be an effective strategy to hinder the progression of VOT and calcification of VSMCs under a high-phosphate environment. DKK proteins specifically inhibit the WNT/β-catenin signaling pathway by interacting with the LRP5/6 coreceptor [33], therefore, our observation that DKK1 inhibits Runx2 induction and calcium deposition in VSMCs clearly underscores a pivotal role of a hyperactive WNT/β-catenin signaling pathway in the pathogenesis of VOT and calcification of VSMCs triggered by high-phosphate. It should be pointed out that the activation of β-catenin signaling through increasing phosphorylation of β-catenin on Ser675 site couldn't be abrogated by DKK1. According to the finding in present study, downregulation of phosphorylated β-catenin (Ser675) through inhibiting PKA or/and PKA1 pathway could be an effective way to partially suppress Runx2 expression, VOT, and in turn, calcification of VSMCs induced by high-phosphate. This notion remains to be verified by further studies.

5. Conclusion

In summery, our present study has demonstrated that Runx2 is a direct transcriptional target of WNT/β-catenin signaling pathway. By inducing this main osteoblast transcription factor, WNT/β-catenin signaling could play a crucial role in promoting VOT and the development and progression of vascular calcification. Furthermore, the concomitant induction of two forms of active β-catenin unveils that the activation of WNT/β-catenin signaling by high-phosphate may be implemented through different pathways. Undoubtedly, further investigation on the function and mechanism of high-phosphate in triggering of WNT/β-catenin signaling activation are hopeful to gain mechanistic insights into VOT and to offer new clues for designing rational strategies to inhibit the progression of vascular calcification in CKD patients.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81170659/H0509 and 31571169/C110201), Natural Science Foundation of Jiangsu Province (BK2012870), Medical Science and Technology Development Foundation of Nanjing City (YKK15193), Qinglan Project of Jiangsu Province (2012), and Six Talent Peaks Project of Jiangsu Province (2014-YY-003) to Weichun He; Key Project of Science and Technology Bureau of Jiangsu Province (BL2013037) to Junwei Yang.

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