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Research Article

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

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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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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-