Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

# Effect of high glucose levels on the calcification of vascular smooth muscle cells by inducing osteoblastic differentiation and intracellular calcium deposition via BMP-2/Cbfα-1 pathway

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Received May 30, 2010; Revision accepted July 25, 2010; Crosschecked Nov. 15, 2010

**Abstract:** In this paper, we investigate the effect and the possible mechanism of high glucose levels on the calcification of human aortic smooth muscle cells (HASMCs). HASMCs were divided into four groups: normal glucose group (NG), osmolality control group (OC), high glucose group (HG, HASMCs culture medium containing 30 mmol/L glucose), and high glucose plus recombinant human Noggin protein (bone morphogenetic protein-2 (BMP-2) antagonist) group (HN). The mRNA levels and the protein expressions of BMP-2 and core binding factor alpha-1 (Cbfα-1) were measured by real-time quantitative polymerase chain reaction (PCR) and Western blot. After induced by 10 mmol/L β-glycerol phosphoric acid, cells were harvested for assessments of alkaline phosphatase (ALP) activities at Days 1, 2, and 3, and intracellular calcium contents at Days 7 and 14, respectively. High glucose levels increased the mRNA levels and the protein expressions of BMP-2 and Cbfα-1 ($P$<0.05). The expression of Cbfα-1 was partially blocked by Noggin protein ($P$<0.05), while BMP-2 was not ($P$>0.05). After being induced by β-glycerol phosphoric acid, high glucose levels increased the ALP activity [(48.63±1.03) vs. (41.42±2.28) U/mg protein, Day 3; $P$<0.05] and the intracellular calcium content [(2.76±0.09) vs. (1.75±0.07) μmol/mg protein, Day 14; $P$<0.05] in a time-dependent manner when compared with the NG group, while the ALP activity could not be blocked by Noggin protein [(48.63±1.03) vs. (47.37±0.97) U/mg protein, Day 3; $P$>0.05]. These results show that high glucose levels can evoke the calcification of HASMCs by inducing osteoblastic *trans*-differentiation and intracellular calcium deposition via the BMP-2/Cbfα-1 pathway, which can be partially blocked by Noggin protein.

**Key words:** Bone morphogenetic protein (BMP), Core binding factor alpha-1 (Cbfα-1), Vascular smooth muscle cell, Noggin protein

**doi:**10.1631/jzus.B1000119 **Document code:** A **CLC number:** R543

## 1 Introduction

Arterial calcification is distinguished into two distinct types: intimal calcification and medial calcification (Doherty *et al.*, 2004). The former is generally recognized to be associated with atherosclerotic plaque lesions, while medial calcification (also called Mönckeberg's calcification) is entirely a different entity. There are several study results consistent with the conclusion that medial calcification is not only a positively regulated process as apparent in the deposition of calcium phosphate mineral, but also an active process resembling osteogenesis. Several investigators have demonstrated that in vitro cultured vascular smooth muscle cells (VSMCs) appear to mineralize in the presence of β-glycerophosphate and undergo a phenotypic differentiation into osteoblast-like cells (Tintut *et al.*, 2000; Steitz *et al.*, 2001; Parhami *et al.*, 2002). Medial calcification is a common pathologic condition that occurs in diabetic patients, and may contribute to increased cardiovascular mortality. It is also considered to be an independent predictor of

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cardiovascular and cerebrovascular events in diabetic patients (Lehto *et al.*, 1996). Recent study has revealed that high glucose levels can enhance the calcification of bovine VSMCs (Chen *et al.*, 2006). However, the underlying mechanisms have not been clarified.

Bone morphogenetic protein-2 (BMP-2) is known as an important osteoinductive regulator and core binding factor alpha-1 (Cbfα-1) downstream has been validated to be the earliest and most specific maker of the osteoblastic phenotype. In this study, we examined the effect of high glucose levels on the calcification of human aortic smooth muscle cells (HASMCs) by detecting the expressions of BMP-2/Cbfα-1, alkaline phosphatase (ALP) activities, and intracellular calcium content, and we used recombinant human Noggin protein (BMP-2 antagonist) to determine the possible role of BMP-2/Cbfα-1 signaling pathway on the calcification of HASMCs induced by high glucose levels.

## 2 Materials and methods

### 2.1 Cell culture

HASMCs (HASMC 6110) and smooth muscle cell medium (SMCM, with 10% (w/v) fetal bovine serum) were purchased from ScienCell (USA). HASMCs were cultured in a 95% air/5% $CO_2$-humidified environment at 37 °C. Cells between Passages 8 to 10 were used for experiments. To investigate the effect of high glucose levels on the expressions of the BMP-2/Cbfα-1 signaling pathway, recombinant human Noggin protein (Peprotech, Rocky Hill, NJ) was used to block the downstream pathway of BMP-2 as a negative regulator. HASMCs were divided into four groups: normal glucose group (NG group, 5.6 mmol/L glucose), osmolality control group (OC group, 5.6 mmol/L glucose plus 24.4 mmol/L D-mannitol), high glucose group (HG group, 30 mmol/L glucose), and high glucose plus 100 ng/ml Noggin protein group (HN group). To determine the effect of high glucose on the intracellular calcium content and ALP activities, calcification-inducing medium, containing 10 mmol/L β-glycerol phosphoric acid by addition of 10 mmol/L sodium pyruvate, was added to the mediums of the above four groups.

### 2.2 Quantitative real-time polymerase chain reaction (PCR) analysis

HASMCs ($10^6$ cells/ml) were cultured in the different conditions in a six-well plate for 24 h. Total RNA was extracted with Trizol (Gibco, Tulsa, OK). A total of 2 μg RNA was reversely transcribed by using ReverTraAce kit (Toyobo, Osaka, Japan) in the PTC-100 Programmable Thermal Controller (MJ Research Inc., Reno, NV). DNA Engine Opticon™ real-time fluorescence quantitative PCR apparatus was used for PCR amplification under 20-μl reaction system. Amplification conditions are as follows: 94 °C for 2 min for a total of 40 cycles, 94 °C for 30 s and 60 °C for 30 s (monitoring fluorescence signal), and preservation at 16 °C. Glyceraldehyde phosphate dehydrogenase (GAPDH) was employed as an internal reference. The TaqMan® probe was labeled at the 5′ end with the fluorescent reporter FAM and at the 3′ end with the fluorescent quencher TAMRA. Intergroup comparison was conducted with delta cycle threshold ($\Delta C_t$) value, which was calculated according to the formula of $\Delta C_t=C_{t,TG}-C_{t,RG}$ ($C_{t,TG}$: $C_t$ of target gene; $C_{t,RG}$: $C_t$ value of reference gene). $C_t$ value was the amount of circulation that was necessary for cDNA to reach exponential amplification. More initial copies of the target gene indicated fewer circulations for exponential amplification and less $\Delta C_t$ value. Sequences of the primer and TaqMan® probe were as follows: (1) BMP-2 primer forward: 5′-TGTATCGCAGG CACTCAGGTC-3′, reverse: 5′-TTCCCACTCGTTT CTGGTAGTTCTT-3′, product length is 136 bp; (2) Cbfα-1 primer forward: 5′-CACTGGCGCTGAA CAAGA-3′, reverse: 5′-CATTCCGGAGCTCAGCA GAATAA-3′, product length is 127 bp; (3) GAPDH primer forward: 5′-GCACCGTCAAGGCTGAGAA C-3′, reverse: 5′-TGGTGAAGACGCCAGTGGA-3′, product length is 138 bp.

### 2.3 Western blot analysis

HASMCs were incubated under different conditions for 72 h. Cells were lysed and sonicated when they reached 80% confluence. Then protein was quantified using the Pierce bicinchoninic acid (BCA) assay system. Aliquots of whole lysates were stored at −20 °C until use. Samples containing 20 μg total proteins were boiled briefly, loaded for sodium

dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes. The blots were incubated with rabbit antibodies against human BMP-2 (1:500 dilution, Boster, Wuhan, China) or Cbfα-1 (1:500 dilution, Boster, Wuhan, China) overnight at 4 °C followed by incubating with peroxidase-conjugated secondary antibody (1:20000 dilution). BMP-2 and Cbfα-1 proteins were detected using an electrochemiluminescence (ECL) Plus kit, and quantitative densitometric analysis was performed on a Bio-Rad Fluor-S MultiImager.

### 2.4 Intracellular calcium deposition quantification

HASMCs ($10^4$ cells/ml) were cultured in six-well plates, and were harvested at Days 7 and 14, respectively. Cells were washed twice with phosphate buffered saline (PBS) and decalcified with 0.6 mol/L HCl at 37 °C for 24 h. The calcium content of the supernatants was determined by the QuantiChrom™ calcium assay kit (Nanjing Chemical Co., China). Then cells were solubilized with a solution of 0.1 mol/L NaOH and 0.1% (w/v) SDS, and the protein contents of the samples were measured using BCA protein assay kit. The calcium content of the cells was normalized to the cellular protein of the culture and expressed as μmol/mg protein.

### 2.5 ALP activity assay

HASMCs ($10^6$ cells/ml) were cultured in six-well plates, and harvested on Days 1, 2, and 3, respectively. Cells growing on six-well plates were washed with PBS twice, and then solubilized with 1% (w/v) Triton X-100 in 0.9% (w/v) NaCl at 4 °C. After being sonicated and centrifugated, the supernatants were assayed for ALP activities using a commercially available assay kit (Nanjing Chemical Co.). Quantitative kinetic determination of cell-associated ALP activity (U/mg protein) was then determined at 30 °C by monitoring the absorbance at 520 nm on a VersaMax™ microplate reader. Results were normalized by paranitrophenol level.

### 2.6 Statistical analysis

Data management was conducted by SPSS 13.0 software. Data were expressed as mean±standard deviation (SD). The paired *t*-test was used for the mean value of intragroup comparison, while the mean comparison among groups was shown with the single factor analysis of variance, multiple comparisons among groups by using LDS method, and repeated measurement data by using repeated measurement analysis of variance. Data were considered to be statistically significant at $P<0.05$.

## 3 Results

### 3.1 Effects of high glucose levels and Noggin on the expressions of BMP-2 and Cbfα-1 mRNA

The levels of BMP-2 and Cbfα-1 mRNA were evaluated by quantitative RT-PCR analysis. The results showed that, when compared with the NG and OC groups, high glucose levels can increase the level of BMP-2 mRNA ($P<0.05$), which is not blocked by Noggin ($P>0.05$) (Figs. 1a and 1c). And when compared with the NG and OC groups, high glucose levels can induce the expression of Cbfα-1 mRNA ($P<0.05$), which is partially blocked by Noggin ($P<0.05$) (Figs. 1b and 1d).

### 3.2 Effects of high glucose levels and Noggin on the expressions of BMP-2 and Cbfα-1 proteins

The expressions of BMP-2 and Cbfα-1 proteins were detected by Western blot assay. The results demonstrated that high glucose levels can elevate the expression of BMP-2 when compared with the NG and OC groups ($P<0.05$), and this is not blocked by Noggin ($P>0.05$) (Figs. 2a and 2c). As well, high glucose levels can induce the expression of Cbfα-1, which is much higher than that of the NG and OC groups ($P<0.05$) and is partially blocked by Noggin ($P<0.05$) (Figs. 2b and 2d).

### 3.3 Effects of high glucose levels and Noggin on the intracellular calcium deposition induced by calcification medium

HASMCs cultured in β-glycerol phosphoric acid-containing calcification mediums were harvested for the assay of the intracellular calcium deposition at Days 0, 7, and 14, respectively. The data showed that high glucose levels can promote intracellular calcium deposition in a time-dependent manner when compared with the NG group [(2.76±0.09) vs. (1.75±0.07) μmol/mg protein, Day 14; $P<0.05$) and the OC group [(2.76±0.09) vs. (1.85±0.17) μmol/mg protein, Day 14; $P<0.05$). This effect is

partially blocked by Noggin [(2.76±0.09) vs. (2.14±0.07) μmol/mg protein, Day 14; $P<0.05$). And there was no difference between the NG and OC groups [(1.75±0.07) vs. (1.85±0.17) μmol/mg protein, Day 14; $P>0.05$) (Table 1).

### 3.4 Effects of high glucose levels and Noggin on the ALP activity

HASMCs were harvested for the ALP activity detection at Days 1, 2, and 3, respectively. The results

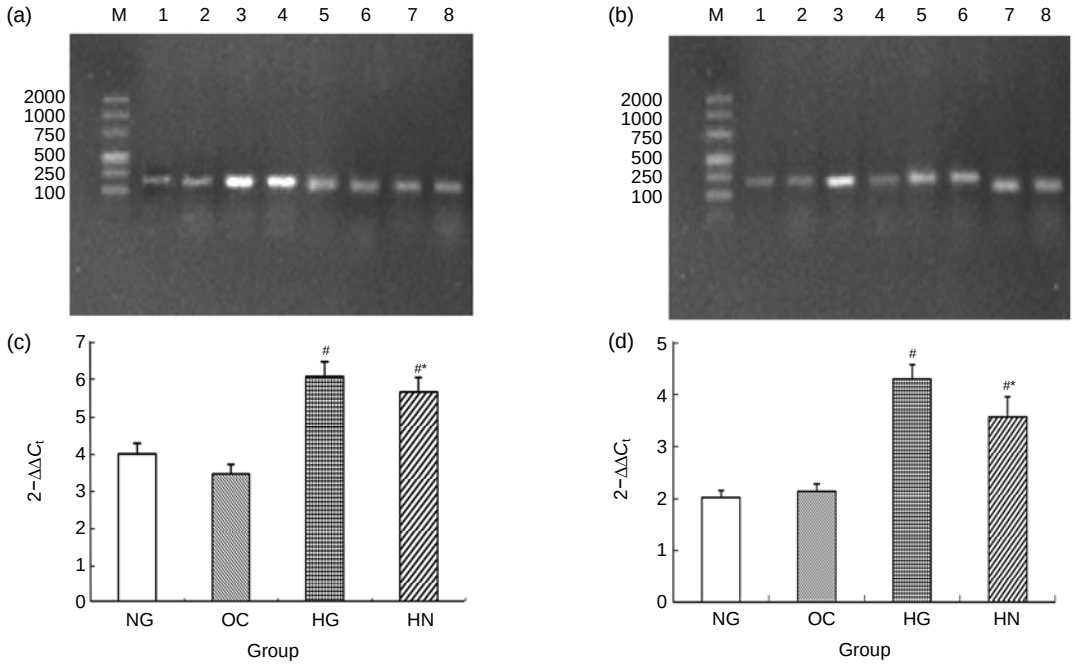


**Fig. 1 Effects of high glucose levels and Noggin on the expressions of BMP-2 and Cbfα-1 mRNA**

HASMCs were cultured in the different conditions for 24 h. Total RNA was extracted and reversely transcribed. BMP-2 (a, c) and Cbfα-1 (b, d) mRNA expressions were measured by real-time PCR. (a, b) M: marker; 1: NG group; 2: OC group; 3: HG group; 4: HN group; 5–8: internal references in different groups; (c, d) Values are expressed as mean±SD ($n$=3). $^{\#}P<0.05$ vs. the control group, $^{*}P<0.05$ vs. the HG group

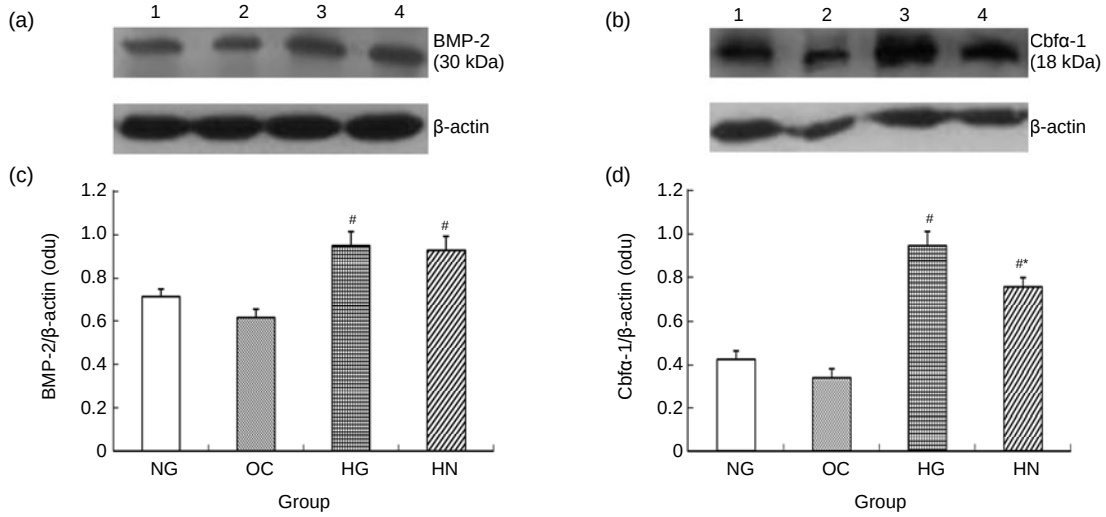


**Fig. 2 Effects of high glucose levels and Noggin on the expressions of BMP-2 and Cbfα-1 proteins**

HASMCs were cultured in the different conditions for 72 h. Cells were harvested and the expressions of BMP-2 (a, c) and Cbfα-1 (b, d) proteins were evaluated by Western blot analysis. (a, b) 1: NG group; 2: OC group; 3: HG group; 4: HN group; (c, d) Values are expressed as mean±SD ($n$=3). $^{\#}P<0.05$ vs. the control group, $^{*}P<0.05$ vs. the HG group

**Table 1 Effects of high glucose levels and Noggin on intracellular calcium deposition**

| Group | Intracellular calcium content (μmol/mg protein) | | |
|---|---|---|---|
| | Day 0 | Day 7 | Day 14 |
| NG | 0.58±0.06 | 1.04±0.07 | 1.75±0.07$^{\#}$ |
| OC | 0.49±0.07 | 1.06±0.07 | 1.85±0.17$^{\#}$ |
| HG | 0.57±0.06 | 1.98±0.06$^{*\#\S}$ | 2.76±0.09$^{*\#\S}$ |
| HN | 0.55±0.06 | 1.58±0.04$^{\#\S}$ | 2.14±0.07$^{\#\S}$ |

Values are expressed as mean±SD ($n$=3). $^{\S}$ $P$<0.05, the HG group vs. the NG group at the same day; $^{\#}$ $P$<0.05, Days 7, 14 vs. Day 0 under the same conditions; $^{*}$ $P$<0.05, the HG group vs. the HN group at the same day

show that high glucose levels can increase the ALP activity in a time-dependent manner [(48.63±1.03) vs. (23.79±0.97) U/mg protein, Day 3; $P$<0.05], wherein the ALP activity of the HG group was much higher than that of the NG group [(48.63±1.03) vs. (41.42±2.28) U/mg protein, Day 3; $P$<0.05], while no significant difference was observed between the HG and HN groups [(48.63±1.03) vs. (47.37±0.97) U/mg protein, Day 3; $P$>0.05]. Thus, ALP activity induced by high glucose levels cannot be blocked by Noggin protein in HASMCs (Table 2).

**Table 2 Effects of high glucose levels and Noggin on the ALP activity**

| Group | ALP activity (U/mg protein) | | |
|---|---|---|---|
| | Day 1 | Day 2 | Day 3 |
| NG | 22.66±1.03 | 34.26±0.96 | 41.42±2.28$^{\#}$ |
| OC | 23.55±1.03 | 34.13±1.48 | 40.17±0.56$^{\#}$ |
| HG | 23.79±0.97 | 39.30±1.20$^{\#\S}$ | 48.63±1.03$^{\#\S}$ |
| HN | 22.75±1.06 | 40.28±1.21$^{\#\S}$ | 47.37±0.97$^{\#\S}$ |

Values are expressed as mean±SD ($n$=3). $^{\S}$ $P$<0.05, the HG group vs. the NG group at the same day; $^{\#}$ $P$<0.05, Days 2, 3 vs. Day 1 under the same conditions

## 4 Discussion

Vascular calcification is a common event in patients with metabolic syndrome, diabetes, and chronic kidney disease (CKD), and is strongly correlated with cardiovascular morbidity and mortality. Recent insights have indicated that the mechanisms of the occurrence and development of the vascular calcification share several similarities with bone formation. A large number of regulatory factors of bone formation and bone structural components (Shanahan *et al.*, 1994; Dhore *et al.*, 2001; Moe and Chen, 2004) were found in the calcified vascular lesions, suggesting that vascular calcification is an actively regulated biological process resembling osteogenesis. VSMCs play an important role in vascular calcification. The main event is that VSMCs undergo osteoblast-like phenotypic differentiation, and these osteoblast-like VSMCs can synthesize and secret a variety of bone formation materials such as ALP, BMP-2, osteocalcin (OC), and matrix vesicles (Mori *et al.*, 1999; Steitz *et al.*, 2001; Shioi *et al.*, 2002).

BMP-2 is one of the members of the transforming growth factor-β (TGF-β) superfamily. It can induce bone marrow pluripotent stem cells to differentiate to osteoblasts, and plays an important role in proliferation and differentiation of cartilage cells and osteoblasts (Attisano and Wrana, 2002). The expression of BMP-2 has been detected in human calcified vessels, and it was also observed that exogenous BMP-2 can increase calcium deposition of bovine VSMCs cultured in vitro (Chen *et al.*, 2006). Cbfα-1 has been identified as the most important transcription factor in the downstream of BMP-2, and plays an important role in the course of bone formation, multipotent mesenchymal stem cells differentiation to osteoblast, osteoblastic differentiation, and osteogenic cells matrix gene expression. It is recognized to be the earliest and most specific osteoblast maker. And Cbfα-1 has been reported to be present in the vascular calcified lesions of hemodialysis patients and non-hemodialysis CKD patients (Chen *et al.*, 2002; Moe *et al.*, 2003). BMP-2 can induce the expression of Cbfα-1 and the two can act synergistically (Lee *et al.*, 2000; Otto *et al.*, 2003). During the process of bone formation, osteoblasts secret ALP and calcium crystals into the extracellular matrix region, and ALP can provide the substrate to facilitate the hydroxyapatite crystallization and increase the local content of phosphoric acid. ALP can also promote the calcification of bovine VSMCs and the release of phosphorus, and therefore, calcification can be blocked by reducing the level of ALP (Shioi *et al.*, 1995).

Compared with non-diabetic patients, the elevated expressions of osteopontin, type I collagen, and ALP, and the augmented calcification in arteries were found in diabetic patients. A variety of factors, such as inflammatory cytokines, oxidative stress,

and advanced glycation end (AGE) product, and signal pathways are involved in the mechanisms of high glucose-promoted vascular calcification. In our study, we observed that high glucose levels might promote vascular calcification by inducing the BMP-2/Cbfα-1 pathway and increasing the ALP activity and intracellular calcium deposition, although in vitro experiments cannot entirely mimic the biological environment in vivo.

Noggin protein is a secreted extracellular glycoprotein that antagonizes the action of BMP-2 by binding to BMPs, and it blocks the interaction between BMPs and their specific receptors, so as to negatively regulate the intracellular signal transduction of BMP-2 and its downstream signaling pathway without affecting BMP-2 biosynthesis (Zhu *et al.*, 2006; Canalis *et al.*, 2003). There is evidence that the TGF-β receptor superfamily (Yuasa and Fukuda, 2008) and receptor-related proteins (Conley *et al.*, 2000) exist in vascular cells such as endothelial cells and smooth muscle cells. Thus, vascular cells are also the target cells of BMP-2. In our experiments, recombinant human Noggin protein was used as the BMP-2 blocking agent, and it was observed not to affect the high glucose-stimulated BMP-2 expression significantly, but to reduce the production of downstream Cbfα-1 and vascular calcification, correspondingly. This is consistent with the mechanisms of Noggin reported by others (Busch *et al.*, 2008; Takayama *et al.*, 2009). Meanwhile, our results also confirmed that high glucose-induced Cbfα-1 expression was regulated by BMP-2, and high glucose levels might induce osteoblastic differentiation and intracellular calcium deposition via the BMP-2/Cbfα-1 pathway. In addition, we found that high glucose-stimulated ALP activity was not blocked by recombinant human protein Noggin. It has been confirmed that β-glycerophosphate, as a phosphate substrate, facilitated the occurrence and progression of mineralization by promoting the release of inorganic phosphate in a dose-dependent manner and increasing the ALP activity (Shioi *et al.*, 1995). Our data showed that Noggin could not reduce high glucose-induced ALP activity. This might indicate that the elevated ALP activity induced by high glucose levels is not regulated primarily by the BMP-2 pathway. Though recombinant human protein Noggin did not reduce high glucose-induced ALP activity, we still cannot deny the effect of Noggin on vascular calcification induced by high glucose.

In conclusion, we observed that high glucose levels increase the expressions of BMP-2 and Cbfα-1, ALP activity, and intracellular calcium deposition in HASMCs cultured in vitro, and that Noggin partially blocked the expression of Cbfα-1 and intracellular calcium deposition. These findings suggest that high glucose levels might evoke the calcification of VSMCs by inducing osteoblastic differentiation and intracellular calcium deposition via the BMP-2/Cbfα-1 pathway in HASMCs in vitro.

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