



# 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> promotes bone formation by promoting nuclear exclusion of the FoxO1 transcription factor in diabetic mice

Received for publication, May 12, 2017, and in revised form, September 27, 2017. Published, Papers in Press, October 17, 2017, DOI 10.1074/jbc.M117.796367

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Edited by Jeffrey E. Pessin

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) is the active form of vitamin D, which is responsible for reducing the risk for diabetes mellitus (DM), decreasing insulin resistance, and improving insulin secretion. Previous studies have shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the activity of FoxO1, which has been implicated in the regulation of glucose metabolism. However, its function and mechanism of action in DM-induced energy disorders and also in bone development remains unclear. Here, using *in vitro* and *in vivo* approaches including osteoblast-specific, conditional FoxO1-knock-out mice, we demonstrate that 1,25(OH)<sub>2</sub>D<sub>3</sub> ameliorates abnormal osteoblast proliferation in DM-induced oxidative stress conditions and rescues the impaired glucose and bone metabolism through FoxO1 nuclear exclusion resulting from the activation of PI3K/Akt signaling. Using alizarin red staining, alkaline phosphatase assay, Western blot, and real-time qPCR techniques, we found that 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes osteoblast differentiation and expression of osteogenic phenotypic markers (*i.e.* alkaline phosphatase (1), collagen 1 (COL-1), osteocalcin (OCN), and osteopontin (OPN)) in a high-glucose environment. Moreover, 1,25(OH)<sub>2</sub>D<sub>3</sub> increased both total OCN secretion and levels of uncarboxylated OCN (GluOC) by phosphorylating FoxO1 and promoting its nuclear exclusion, indicated by Western blot and cell immunofluorescence analyses. Taken together, our findings confirm that FoxO1 is a key mediator involved in glucose homeostasis and indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub> improves glucose metabolism and bone development via regulation of PI3K/Akt/FoxO1/OCN pathway.

Diabetes mellitus (DM)<sup>3</sup> is a chronic metabolic disorder that affects hundreds of millions of individuals. Apart from renal and cardiovascular complications, DM-induced hyperglycemia adversely affects bone metabolism, such as foot syndrome and Charcot neuroarthropathy (2–4). DM-induced oxidative stress causes osteoporosis, another skeletal complication that increases bone fragility (5–7). Studies suggested that insufficient peak bone mass and impaired bone formation in type 1 diabetes mellitus DM and the poor bone quality in type 2 DM (5, 8, 9) were proposed as major contributing factors in bone fragility. In addition, DM is also considered as one of contraindications to dental implant (10). Patients with well-controlled DM are acceptable, whereas those with poorly controlled DM are always declined for implant therapy. Consistent with this concern, lots of animal studies also demonstrated that delayed bone-implant healing and implant osseointegration were related to inadequate glycemia control (11–13).

Cellular oxidative stress and reactive oxygen species are suggested to be involved in DM development. FoxO (forkhead homeobox type O) transcription factors are regarded as mediators to oxidative stress because they can be regulated by reactive oxygen species (14). FoxO transcription factors induce apoptosis, stress resistance, and senescence (15, 16). As a member of FOXO transcription factors, FoxO1 orchestrates transcriptional actions regulating glucose metabolism (17). In addition, FoxO1 is highly expressed in skeleton that has, during the last few years, been identified as a transcriptional link between the skeleton and pancreas to regulate glucose homeostasis (18). In osteoblasts, FoxO1 regulates energy metabolism and inhibits insulin secretion and sensitivity. In the feedback regulation, insulin suppresses FoxO1 activity and promotes its nuclear exclusion by activating PI3K/Akt signaling pathway (19, 20). Sequence alignment shows that FoxOs proteins have highly conserved regions, including the N-terminal region containing an Akt phosphorylation site (21), through a combination of which FoxO1 can be phosphorylated by serine–threonine kinase Akt.

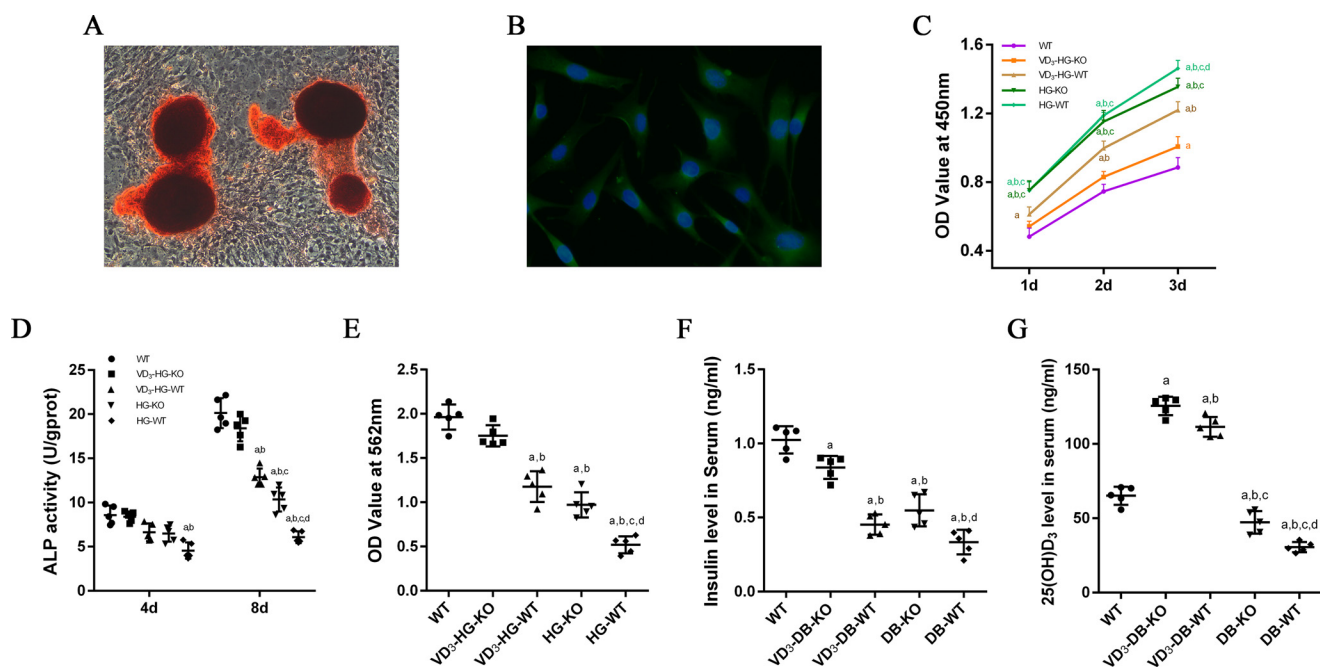
1,25(OH)<sub>2</sub>D<sub>3</sub>, an active form of vitamin D, is responsible for the balance of calcium and phosphorus, bone mineralization,

This work was supported by National Natural Science Foundation of China Grants NSFC 81400543 and NSFC 81571008 and Outstanding Scholars Research Funding of Sichuan University (2017SCU04A21). The authors declare that they have no conflicts of interest with the contents of this article.

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<sup>3</sup> The abbreviations used are: DM, diabetes mellitus; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; COL-1, collagen 1; OCN, osteocalcin; GluOC, uncarboxylated OCN; OPN, osteopontin; ALP, alkaline phosphatase; tOCN, total osteocalcin; ucOCN%, the percentage of uncarboxylated osteocalcin; VDR, vitamin D receptor; OB, osteoblast;  $\alpha$ MEM,  $\alpha$  minimum Eagle's medium.



**Figure 1. 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) decreases abnormal proliferation and promotes osteogenic differentiation of osteoblasts in high-glucose environment.** A, alizarin red staining of primary osteoblasts after 21-day osteogenic induction. B, immunofluorescent staining of OCN in osteoblasts. Alizarin red-positive nodules and obvious expression of OCN proves a successful isolation and culture of primary osteoblasts. C, cell viability determined by the CCK-8 assay at 1, 2, and 3 days in different groups (*n* = 6 specimens/group). D, cell differentiation assessed by ALP activity at 4 (4d) and 8 days (8d) (*n* = 5 specimens/group). E, quantification of mineralization nodules in different groups (*n* = 5 specimens/group). F and G, serum insulin (F) and serum 25(OH)<sub>2</sub>D<sub>3</sub> (G) were detected by ELISA (*n* = 5 specimens/group). The data are presented as means ± S.D. a, *p* < 0.05 for WT versus others; b, *p* < 0.05 for VD<sub>3</sub>-HG(DB)-WT or HG(DB)-KO or HG(DB)-WT versus VD<sub>3</sub>-HG(DB)-KO; c, *p* < 0.05 for HG(DB)-KO or HG(DB)-WT versus VD<sub>3</sub>-HG(DB)-WT; d, for HG(DB)-WT versus HG(DB)-KO. DB represents diabetes mellitus, and HG represents high glucose.

and hormonal release (22). There is evidence showing a close relationship between DM and vitamin D. Vitamin D supplementation can reduce the risk for DM and insulin resistance (23, 24) and improve insulin secretion, insulin sensitivity, and HbA1c (25). Our previous research demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> could promote insulin secretion and bone remodeling, maintain glucose homeostasis, and ameliorate implant osseointegration capacity in diabetic rats (26). The subsequent research<sup>4</sup> further suggested that 1,25(OH)<sub>2</sub>D<sub>3</sub> inactivated osteoblastic FoxO1 and promoted its nuclear exclusion in high-glucose environment, indicating that 1,25(OH)<sub>2</sub>D<sub>3</sub> might play its role through FoxO1 inactivation.

Given FoxO1 has been regarded as a main target of vitamin D action in other tissues, including skeletal muscle (27), we investigated whether it fulfills its regulatory effect, at least in part, through its osteoblastic expression. In this report, we show herein that conditional knock-out FoxO1 in osteoblasts favors glucose metabolism and bone formation. Of note, 1,25(OH)<sub>2</sub>D<sub>3</sub> prevents DM-induced bone loss and ameliorates osteoblastic capacity through PI3K/Akt/FoxO1/OCN pathway. That is, 1,25(OH)<sub>2</sub>D<sub>3</sub> inactivates FoxO1 activity and promotes OCN expression and its uncarboxylated level, thus helping restrain the deleterious effects induced by high glucose, indicating that 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment and FoxO1 inhibition might be a novel therapeutic approach to enhance bone formation.

## Results

### 1,25(OH)<sub>2</sub>D<sub>3</sub> can rescue abnormal proliferation caused by high glucose

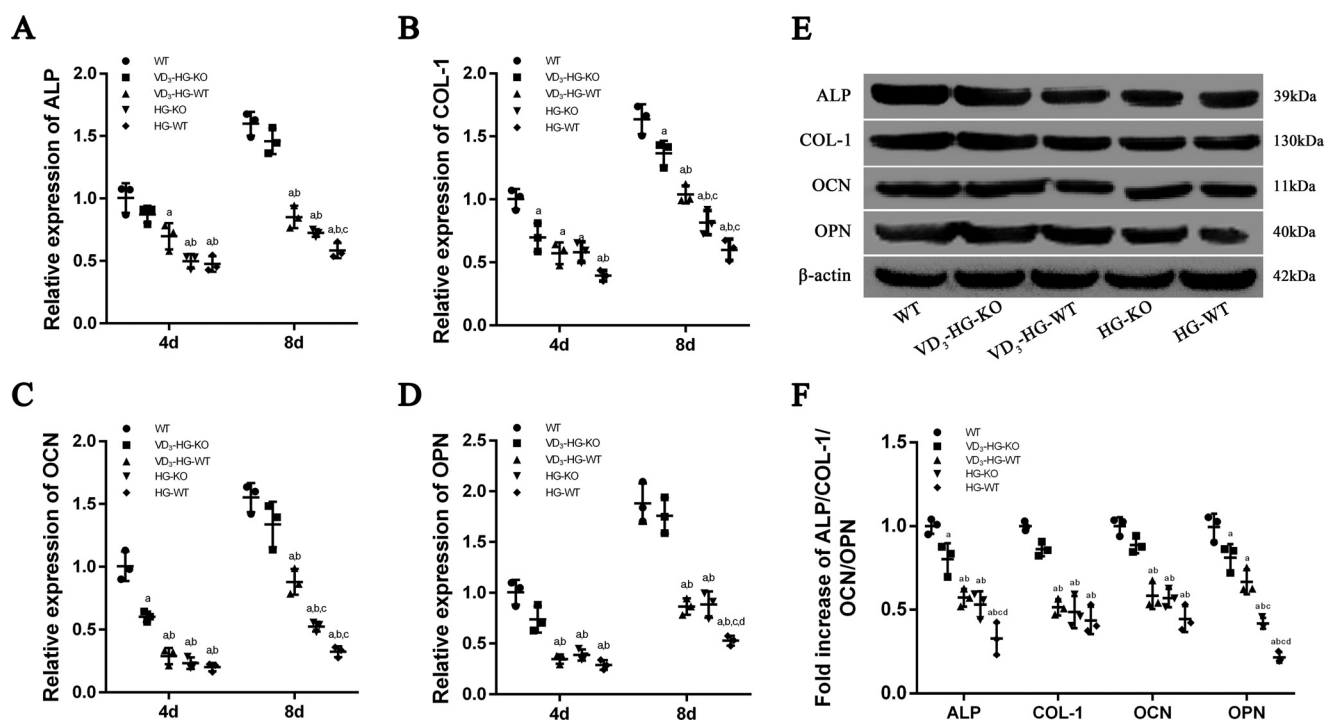
The successful isolation and culture of osteoblasts can be proved by alizarin red staining (Fig. 1A) and OCN (Fig. 1B) immunofluorescence. To evaluate the role of conditional FoxO1 knock-out in osteoblasts (FoxO1<sub>OB</sub><sup>-/-</sup>) and 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment on osteoblasts growth in high-glucose environment, Cell Counting Kit-8 was conducted to detect cell proliferation at 1, 2, and 3 days, respectively. As shown in Fig. 1C, high glucose promoted cell proliferation throughout the whole experimental procedure. When compared with HG-WT group, FoxO1 knock-out reduced cell viability at 3 days in HG-KO group (*p* < 0.05). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly reduced cell viability in the VD<sub>3</sub>-HG-WT and VD<sub>3</sub>-HG-KO groups, especially VD<sub>3</sub>-HG-KO group (*p* < 0.05).

### Improved osteogenic differentiation capacity and mineralization in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated FoxO1<sub>OB</sub><sup>-/-</sup> osteoblasts in high-glucose environment

To investigate the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and FoxO1<sub>OB</sub><sup>-/-</sup> on osteogenic differentiation, alkaline phosphatase (ALP), a marker of early osteogenesis, was detected at 4 and 8 days after 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (28) (Fig. 1D). The results showed that high glucose significantly reduced ALP activity in HG-WT group, whereas FoxO1<sub>OB</sub><sup>-/-</sup> could improve ALP activity in HG-KO group when compared with HG-WT group (*p* < 0.05). 1,25(OH)<sub>2</sub>D<sub>3</sub> up-regulated ALP activity in VD<sub>3</sub>-HG-KO and

<sup>4</sup> Y. Xiong, Y. Zhang, N. Xin, Y. Yuan, Q. Zhang, P. Gong, and Y. Wu, unpublished results.

## 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes bone formation with FoxO1 inactivation



**Figure 2.** 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) increases the expression of osteogenic phenotype in high-glucose environment. A–D, relative expressions of ALP (A), COL-1 (B), OCN (C), and OPN (D) detected at day 4 (4d) and day 8 (8d) by RT-qPCR analysis. All data were normalized to GAPDH. The data are presented as means ± S.D. (n = 3 specimens/group). E and F, quantification of protein levels of ALP, COL-1, OCN, and OPN determined using Western blot (n = 3 specimens/group). a, p < 0.05 for WT versus others; b, p < 0.05 for VD<sub>3</sub>-HG-WT or HG-KO or HG-WT versus VD<sub>3</sub>-HG-KO; c, p < 0.05 for HG-KO or HG-WT versus VD<sub>3</sub>-HG-WT; d, for HG-WT versus HG-KO. DB represents diabetes mellitus, and HG represents high glucose.

VD<sub>3</sub>-HG-WT group ( $p < 0.05$ ). Notably, in high glucose condition, the expression of ALP activity in VD<sub>3</sub>-HG-KO mice is significantly higher than other groups ( $p < 0.05$ ), which shows no significant difference compared with the WT group ( $p > 0.05$ ).

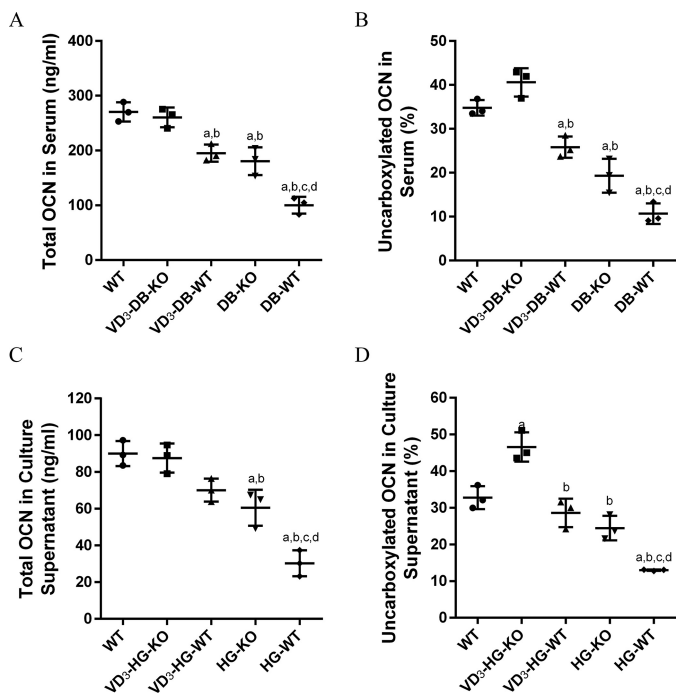
To elucidate the capacity of osteoblastic mineralization in different groups, alizarin red staining was conducted to assess late osteogenic differentiation. As shown in Fig. 1E, after 3-week osteogenic induction, the OD value of alizarin red staining in HG-KO group was higher than that of the HG-WT group ( $p < 0.05$ ). 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment promoted osteoblastic mineralization in the VD<sub>3</sub>-HG-KO and VD<sub>3</sub>-HG-WT groups. In addition, the VD<sub>3</sub>-HG-KO group showed no statistical difference in OD value when compared with the WT group ( $p > 0.05$ ).

The results of the *in vivo* study showed that FoxO1<sub>OB</sub><sup>-/-</sup> promoted insulin secretion (Fig. 1F). Although the expression of insulin level in VD<sub>3</sub>-DB-KO mice is lower than that in WT mice ( $p < 0.05$ ), it is much higher than other three groups in high glucose condition ( $p < 0.05$ ), which might be an explanation for the pro-osteogenic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblasts in as high-glucose environment. 25(OH)D<sub>3</sub> acts as the indicator of VD<sub>3</sub> nutritional status. We measured 25(OH)D<sub>3</sub> by ELISA kit, and the results in Fig. 1G showed that the serum 25(OH)D<sub>3</sub> level decreased significantly in untreated DB-WT mice when compared with other groups ( $p < 0.05$ ). A slight increase of 25(OH)D<sub>3</sub> was detected in DB-KO mice, indicating that FoxO1 knock-out favored serum 25(OH)D<sub>3</sub> ( $p < 0.05$ ). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> application resulted in a notable higher

level of 25(OH)D<sub>3</sub> in VD<sub>3</sub>-DB-KO and VD<sub>3</sub>-DB-WT mice when compared with other three groups ( $p < 0.05$ ).

### Increased osteogenic-related genes and proteins in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated FoxO1<sub>OB</sub><sup>-/-</sup> osteoblasts in high-glucose environment

To further elucidate the osteogenic capacity of osteoblasts subjected to different treatment, we analyzed mRNA and protein expressions of osteogenic phenotype at 4 and 8 days, including ALP, COL-1, OCN, and OPN (29) (Fig. 2, A–D). When compared with WT cells, the mRNA levels of ALP, COL-1, OCN, and OPN in HG-WT cells reduced by ~52.7, 60.7, 80.0, and 71.3% at 4 days and by 63.5, 63.3, 79.1, and 71.9% at 8 days ( $p < 0.05$ ). Moreover, VD<sub>3</sub>-HG-WT cells showed an increase level of ALP, COL-1, OCN, and OPN at 8 days when compared with HG-WT group ( $p < 0.05$ ). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> showed a remarkably beneficial effect of pro-osteogenesis in VD<sub>3</sub>-HG-KO, with no statistical difference in the expression of ALP, OCN, and OPN when compared with the WT group. Western blot analysis was also carried out to verify the protein level of osteogenic markers at 8 days. As shown in Fig. 2 (E and F), when compared with WT group, high glucose reduced the expression of ALP, COL-1, OCN, and OPN in HG-WT ( $p < 0.05$ ). 1,25(OH)<sub>2</sub>D<sub>3</sub> up-regulated these osteogenic markers in the VD<sub>3</sub>-HG-WT group. The results showed that there was no difference in COL-1 and OCN protein level between WT and VD<sub>3</sub>-HG-KO group ( $p > 0.05$ ). Generally, the results of Western blot analysis were consistent with the RT-qPCR assays.



**Figure 3.** 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) treatment and FoxO1 knock-out increases total osteocalcin and uncarboxylated osteocalcin level in serum and cellular supernatant by ELISA. A and B, total (A) and uncarboxylated OCN (%) (B) in serum. C and D, total (C) and uncarboxylated OCN (%) (D) in culture supernatant. The data are presented as means  $\pm$  S.D. ( $n = 3$  specimens/group). a,  $p < 0.05$  for WT versus others; b,  $p < 0.05$  for VD<sub>3</sub>-HG(DB)-WT or HG(DB)-KO or HG(DB)-WT versus VD<sub>3</sub>-HG(DB)-KO; c,  $p < 0.05$  for HG(DB)-KO or HG(DB)-WT versus VD<sub>3</sub>-HG(DB)-WT; d, for HG(DB)-WT versus HG(DB)-KO. DB represents diabetes mellitus, and HG represents high glucose.

#### FoxO1 might mediate the metabolic actions of uncarboxylated osteocalcin *in vitro* and *in vivo*

To understand the potential mechanism of 1,25(OH)<sub>2</sub>D<sub>3</sub> regulatory process, we assayed total osteocalcin (tOCN) and the percentage of uncarboxylated osteocalcin (ucOCN%) *in vivo* and *in vitro* study. Mouse serum (Fig. 3, A and B) was harvested 2 months after surgery, and culture supernatant (Fig. 3, C and D) was collected after 8-day treatment in different conditions. Both of the *in vivo* and *in vitro* studies showed that tOCN and ucOCN% in hyperglycemia was significant lower than that in normal condition ( $p < 0.05$ ). The expression of tOCN and ucOCN% in VD<sub>3</sub>-DB-KO mice increased by 2.6- and 3.8-fold, respectively, when compared with DB-WT mice ( $p < 0.05$ ), which showed no significant difference with WT mice ( $p < 0.05$ ). In addition, the level of tOCN and ucOCN% in VD<sub>3</sub>-DB-WT and DB-KO mice slightly increased when compared with DB-WT mice. *In vitro* study, the results were consistent with *in vivo* study. Of note, the ucOCN% in VD<sub>3</sub>-HG-KO was even much higher than WT group (Fig. 3D,  $p < 0.05$ ).

#### 1,25(OH)<sub>2</sub>D<sub>3</sub> induced a time-dependent Akt and FoxO1 phosphorylation

To further investigate the role of FoxO1 in 1,25(OH)<sub>2</sub>D<sub>3</sub> regulation process, we evaluated the expression of p-Akt/t-Akt and p-FoxO1/t-FoxO1 at 0, 30, 60, 120, 240, 480, and 720 min after the treatment of 1,25(OH)<sub>2</sub>D<sub>3</sub> using primary WT osteoblasts in a high-glucose environment (Fig. 4, A and B). p-Akt is the active form of Akt, whereas p-FoxO1 is the inactive form

of FoxO1. When compared with 0 min, p-Akt/t-Akt ratio increased by 81.5% at 30 min, and p-FoxO1/t-FoxO1 ratio increased by 44.6% at 60 min, respectively ( $p < 0.05$ ). The ratio of p-Akt/t-Akt reached its peak at 2 h after treatment and remained in a relatively high level in the next 10 h. The results suggested that p-Akt/p-FoxO1 might be involved in the regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> on glucose and bone metabolism.

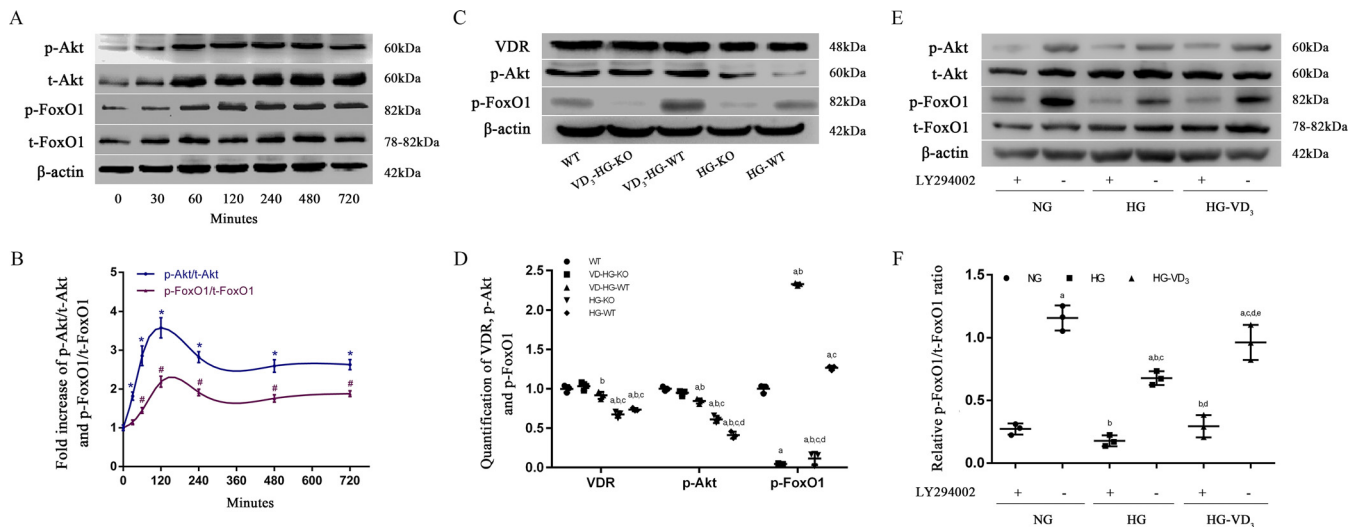
#### 1,25(OH)<sub>2</sub>D<sub>3</sub> promoted osteogenesis by activating vitamin D receptor (VDR)/PI3K/Akt signaling pathway *in vivo* and *in vitro*

To confirm the role of VDR/PI3K/Akt pathway in the regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> on glucose and bone metabolism, we examined the expression of VDR, p-Akt, p-FoxO1 using Western blot (Fig. 4, C–F), immunofluorescence (Fig. 5), and immunohistochemistry (Fig. 6). Given that the p-Akt/t-Akt ratio reached its peak at 2 h after the treatment of 1,25(OH)<sub>2</sub>D<sub>3</sub>, the whole cell protein was collected after a 2-h treatment of 1,25(OH)<sub>2</sub>D<sub>3</sub> and subjected to Western blot analysis. The results manifested that high glucose inhibited the expression of VDR and p-Akt while promoting FoxO1 phosphorylation. 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment dramatically enhanced VDR expression by 53.0% in VD<sub>3</sub>-HG-KO group and 24.6% in VD<sub>3</sub>-HG-WT group (Fig. 4, C and D;  $p < 0.05$ ). In addition, remarkable increase of p-Akt was detected in VD<sub>3</sub>-treated groups when compared with the non-treated high glucose groups ( $p < 0.05$ ). 1,25(OH)<sub>2</sub>D<sub>3</sub> increased FoxO1 phosphorylation in VD<sub>3</sub>-HG(DB)-WT group by 133 and 106.3% as compared with WT and HG-WT group, respectively. LY294002, inhibitor of PI3K, remarkably reduced p-FoxO1/FoxO1 ratio, demonstrating that FoxO1 was the downstream of Akt and could be phosphorylated by p-Akt (Fig. 4, E and F).

In cell immunofluorescent staining, 1,25(OH)<sub>2</sub>D<sub>3</sub> enhanced VDR and p-Akt expression in VD<sub>3</sub>-HG-KO, with no obvious difference compared with WT group (Fig. 5, A and B). A relatively weaker green fluorescence was detected in HG-KO and HG-WT groups. High glucose obviously promoted FoxO1 expression and its nuclear location, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> down-regulated FoxO1 by promoting its nuclear exclusion. Results in immunohistochemistry were consistent with immunofluorescence. Positive staining of VDR and p-Akt was detected mainly in osteoblasts grown along the bone surface in WT, VD<sub>3</sub>-HG-KO, and VD<sub>3</sub>-HG-WT mice (Fig. 6, B–G), whereas the staining of VDR and p-Akt in the HG-KO and HG-WT groups was weaker than in the WT group ( $p < 0.05$ ). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> also attenuated the high expressed FoxO1 induced by diabetes *in vivo* study.

To verify the pro-osteogenic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblasts was achieved through PI3K/Akt signaling pathway, we applied PI3K inhibitor LY294002 to block PI3K/Akt pathway in WT osteoblasts treated with NG, HG, and VD<sub>3</sub>-HG separately (Fig. 7, A–D). 1,25(OH)<sub>2</sub>D<sub>3</sub> ameliorated the reduced expression of the ALP, COL-1, OCN, and OPN in high-glucose medium, although LY294002 abolished the pro-osteogenic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblasts, with no difference with HG-LY294002(+) group ( $p > 0.05$ ). The evidence demonstrated that the pro-osteogenic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblasts was achieved through PI3K/Akt signaling pathway.

# 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes bone formation with FoxO1 inactivation



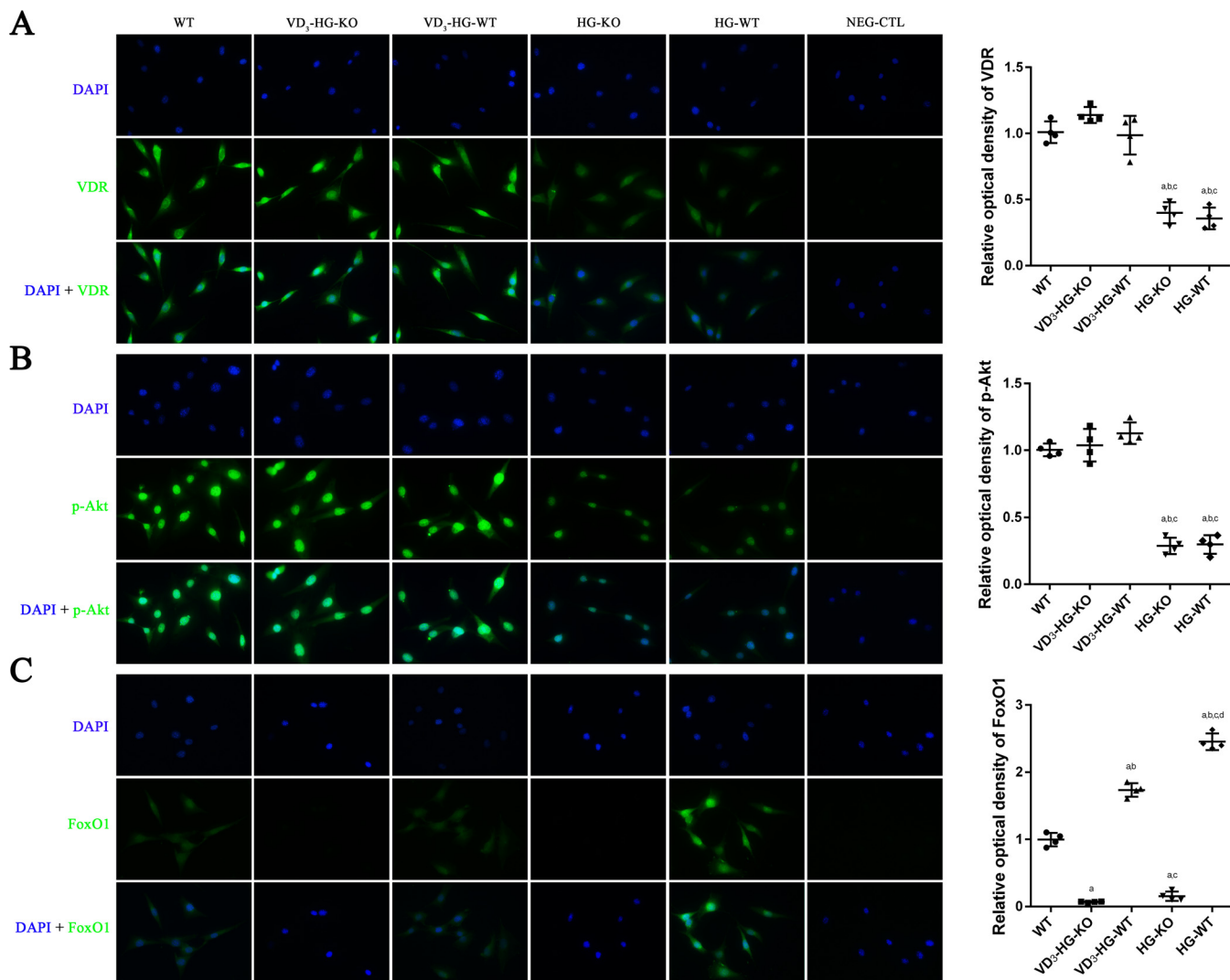
**Figure 4.** PI3K/Akt/FoxO1 signaling pathway involved in the regulatory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) on osteoblasts. A and B, changes of protein levels of p-Akt, t-Akt, p-FoxO1, and t-FoxO1 (A) and the fold increase of p-Akt/t-Akt, p-FoxO1/t-FoxO1 at 0, 30, 60, 120, 240, 480, and 720 min (B) were determined by Western blot. C and D, quantification of VDR, p-Akt, and p-FoxO1 were detected by Western blot after 2-h VD<sub>3</sub> treatment. E and F, changes of p-Akt/t-Akt (E) and quantification of p-FoxO1/FoxO1 (F) determined by Western blot. Whole cell protein was harvested with or without a 2-h incubation of PI3K inhibitor LY294002 (50 mol/liter). The data are presented as means ± S.D. (n = 3 specimens/group). A and B, \*, p < 0.05 for 0 min versus other time points in p-Akt/t-Akt; #, p < 0.05, for 0 min versus other time points in p-FoxO1/t-FoxO1. C and D, a, p < 0.05 for WT versus others; b, p < 0.05 for VD<sub>3</sub>-HG-WT or HG-KO or HG-WT versus VD<sub>3</sub>-HG-KO; c, p < 0.05 for HG-KO or HG-WT versus VD<sub>3</sub>-HG-WT; d, for HG-WT versus HG-KO. E and F, a, p < 0.05 for NG-LY294002(+) versus others; b, p < 0.05, for HG-LY294002(±) or HG-VD<sub>3</sub>-LY294002(±) versus NG-LY294002(-); c, p < 0.05, for HG-LY294002(-) or HG-VD<sub>3</sub>-LY294002(±) versus HG-LY294002(+); d, p < 0.05, for HG-VD<sub>3</sub>-LY294002(±) versus HG-LY294002(-); e, p < 0.05, for HG-VD<sub>3</sub>-LY294002(-) versus HG-VD<sub>3</sub>-LY294002(+). HG represents high glucose, and NG represents normal glucose.

## Discussion

The oxidative stress caused by DM has negative effects on bone quality and strength (30). It has recently been suggested that FoxO1 is linked to prevent osteoblasts from oxidative stress (31, 32). Based on this evidence, we hypothesize that this molecule may mediate bone metabolism in DM. Moreover, the beneficial effects of vitamin D on glucose metabolism and bone formation may be achieved through this transcription factor. To support our hypothesis, we present genetic evidence showing that 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes glucose metabolism<sup>4</sup> and bone formation through, at least in part, inactivation of FoxO1 and the aroused elevation of OCN and uOCN%. Through this mechanism, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the abnormal proliferation induced by high glucose and promotes osteoblastic differentiation and bone formation. High glucose interferes osteoblastic gene expression (33), thus inhibiting the process of osteoblast maturation and bone mineralization. These results are consistent with extensive evidence of Almeida and co-workers (34), who suggested that FoxOs resulted in the loss of cancellous bone mass, whereas deletion of FoxOs could ameliorate the adverse effects resulted from DM-induced oxidative stress, thereby promoting bone formation. They further demonstrated that this beneficial effect on bone formation in FoxO-deficient mice was due to up-regulation of Wnt/ $\beta$ -catenin signaling and cyclin D1 expression (35). However, these observations are challenged by some other publications suggesting that conditional knock-out of FoxOs increased skeletal oxidative stress and osteoblast apoptosis and thereby attenuated bone formation rate and bone mass (31). Because FoxOs family has four isoforms, and each one has its own functions, part of this discrepancy can be attributed to different transgenic strategies. In addition, different mice strains and its disease models may be other possibilities.

As a specific secreted protein in osteoblast, OCN has recently been regarded as a hormone that is involved in glucose and energy metabolism. Two forms of OCN exists in circulation:  $\gamma$ -carboxylated GlaOC and uncarboxylated GluOC. Karsenty and co-workers (36) first reported that OCN-null mice exhibited severe damage in glucose metabolism, indicating that GluOC functions as a hormone (37, 38). In this study, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment facilitated secretion of total OCN and GluOC% level. Particularly, when compared with DB-WT mice, FoxO1<sub>OB</sub><sup>-/-</sup> diabetic mice without any treatment showed a significant increase in OCN and GluOC%, which may be explained by the elevated insulin in circulation. Evidence showed that OCN knock-out reduced  $\beta$ -cell number, insulin secretion, and impaired glucose metabolism (37). In addition, inactivation of GPRC6A, receptor of GluOC, especially in  $\beta$ -cells resulted in glucose intolerance caused by a notable decrease in  $\beta$ -cell number and insulin secretion in circulation (39), suggesting GluOC directly activates GPRC6A and triggers  $\beta$ -cell proliferation and insulin production (40). Our work was also consistent with the result of Kousteni and co-workers (20), who claimed that FoxO1 could bind to OCN promoter and inhibit its expression. Taking all the evidence published and what we found in our study, we suggest that oxidative stress induced by hyperglycemia *in vivo* or high glucose *in vitro* stimulates FoxO1 activity and promotes its nuclear location, which inhibits the expression of OCN and its GluOC level. Conversely, FoxO1 knock-out in osteoblasts or 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced FoxO1 inactivation could rescue the impairment via favoring OCN production and GluOC level.

In addition, because insulin receptors have been shown to be expressed in osteoblasts (41), and evidence also showed the beneficial effects of insulin on osteoblasts production and dif-



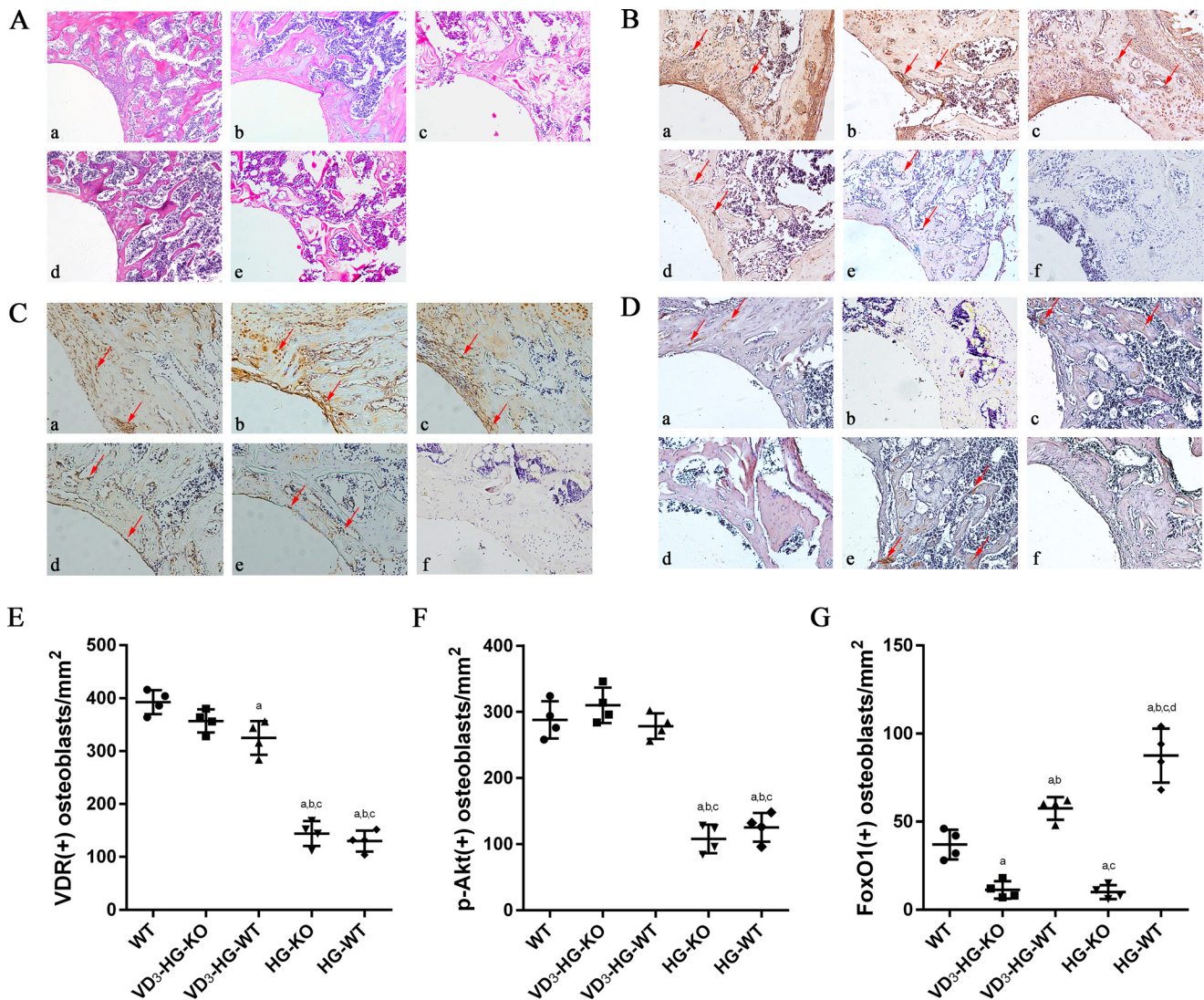
differentiation (42–44), the elevated insulin level mediated by GluOC in our study could, in turn, stimulate insulin signaling in osteoblasts, thereby 1) promoting osteoblast differentiation and bone matrix (45) and 2) inhibiting FoxO1 activation (46), which forms a feed forward regulatory loop.

*In vitro* studies have shown that high glucose weakened osteoblast response to 1,25(OH)<sub>2</sub>D<sub>3</sub> and indirectly down-regulated VDR expression (47). Likewise, our previous study also showed that high glucose attenuated the expression of insulin receptor and VDR, whereas these adverse effects can be reversed by 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (48). In this study, we found that 1,25(OH)<sub>2</sub>D<sub>3</sub> rescued the decreased VDR expression induced by high glucose. In addition, we verified that this beneficial effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on bone metabolism and glucose homeostasis under oxidative stress condition were achieved through a combination with VDR. Phosphorylation is one of the main post-translational modifications of FoxO1, the process of which can be induced by the activation of PI3K/Akt signaling (49). Based on this evidence, we speculated that the effect of

1,25(OH)<sub>2</sub>D<sub>3</sub> on FoxO1/OCN expression might be achieved through PI3K/Akt signaling. In our study, Western blot and immunofluorescence staining verified the involvement of PI3K/Akt signaling in the regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub>. In other words, 1,25(OH)<sub>2</sub>D<sub>3</sub> combined VDR in osteoblasts subsequently activated and phosphorylated Akt, and then the increased p-Akt phosphorylated FoxO1 and promoted its nuclear exclusion, which indirectly promoted OCN expression and uOCN% level in the circulation in hyperglycemia environment.

As for Akt phosphorylation, professor Huhtakangas *et al.* (50) suggested that the anti-apoptosis effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> might include a pertussis toxin-sensitive G protein upstream of PI3K activation, which interacts with the PI3K catalytic subunit, resulting in stimulation of PI3K. In some other cases, 1,25(OH)<sub>2</sub>D<sub>3</sub> has also been reported to associate with the activation of some novel membrane receptors that bind to a G protein (51, 52). This evidence might explain how 1,25(OH)<sub>2</sub>D<sub>3</sub> phosphorylates Akt, but further research is needed to elucidate the details of the mechanism.

## 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes bone formation with FoxO1 inactivation



**Figure 6. Histomorphometric analysis of trabecular changes and immunochemistry of VDR/p-Akt/FoxO1.** A, HE staining of the trabecular near the implant ( $\times 100$ ). B–G, IHC staining and semiquantification for VDR (B and E), p-Akt (C and F), and FoxO1 (D and G) around implant in different groups ( $\times 100$ ). Positive osteoblasts are marked with red arrows. A–D, panels a, WT; panels b, 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>)-DB-KO; panels c, VD<sub>3</sub>-DB-WT; panels d, DB-KO; panels e, DB-WT; panels f, negative control. E–G, data are presented as means  $\pm$  S.D. ( $n = 3$  specimens/group). a,  $p < 0.05$  for WT versus others; b,  $p < 0.05$  for VD<sub>3</sub>-HG-WT or HG-KO or HG-WT versus VD<sub>3</sub>-HG-KO; c,  $p < 0.05$  for HG-KO or HG-WT versus VD<sub>3</sub>-HG-WT; d, for HG-WT versus HG-KO.

It should be noted that VD<sub>3</sub>-HG-KO showed better pro-osteogenic effect than HG-KO, indicating that there existed other pathways involving in the regulation process of 1,25(OH)<sub>2</sub>D<sub>3</sub> on bone and glucose metabolism. Apart from FoxO1/OCN pathway in osteoblasts, VDR found in pancreatic  $\beta$ -cells, and 1 $\alpha$ -hydroxylase expressed in pancreatic islet tissues (53, 54) also suggests that vitamin D directly influences insulin production. On the other hand, vitamin D insufficient causes low muscle mass and muscle weakness. Given the cross-talk between muscle and bone (55, 56), 1,25(OH)<sub>2</sub>D<sub>3</sub> may also regulate bone metabolism indirectly via affecting skeletal muscle. This evidence may account for the better effects found in the VD<sub>3</sub>-HG-KO group in our study.

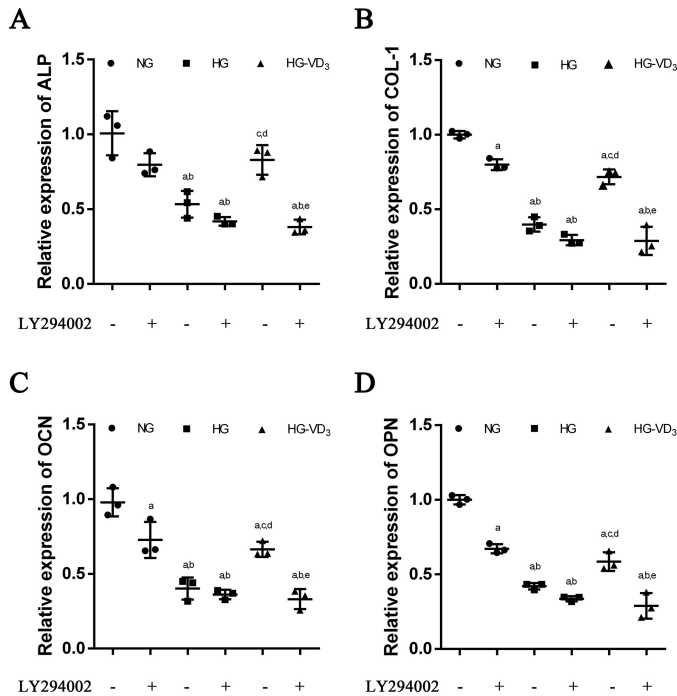
In summary, the results of this study expand our understanding of the endocrine function of the skeleton in a new perspective. We show that 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment rescues the energy and bone metabolism disorder induced by diabetes. We further confirm that 1,25(OH)<sub>2</sub>D<sub>3</sub> activates PI3K/Akt through combi-

nation with VDR *in vivo* and *in vitro*, which subsequently phosphorylates FoxO1 and thus promotes GlaOC and GluOC secretion. In addition, sole FoxO1 knock-out in osteoblasts without 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment can, in part, promote osteogenic phenotype and ameliorate oxidative stress. Our data demonstrate that FoxO1 is a critical mediator of glucose homeostasis and bone metabolism and suggest that FoxO1 may be a potential target for treatment of diabetes and diabetes-induced osteoporosis.

### Experimental procedures

#### Animal study

FoxO1<sup>OB<sup>-/-</sup></sup> mice were obtained by crossing FVB.129S6 (Cg)-FoxO1<sup><tm1Rdp>/J</sup> (FoxO1<sup>fl/fl</sup>) mice and B6N.FVB-Tg-(BGLAP-cre)1Clem/J (Cre<sup>+/-</sup>) mice (57, 58), which were both purchased from Jackson Laboratories. Phenotypes of mice were identified using PCR. FoxO1<sup>fl/fl</sup> mice were first crossed with



**Figure 7. PI3K/Akt inhibitor LY294002 blocks the pro-osteogenic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) on osteoblasts in a high-glucose environment.** A–D, relative expressions of ALP (A), COL-1 (B), OCN (C), and OPN (D) detected at day 8 by RT-qPCR analysis. The cells were pretreated with a 2-h incubation of PI3K inhibitor LY294002 (50 mol/liter). All data were normalized to GAPDH. The data are presented as means ± S.D. (*n* = 3 specimens/group). *a*, *p* < 0.05 for WT versus others; *b*, *p* < 0.05 for VD<sub>3</sub>-HG-WT or HG-KO or HG-WT versus VD<sub>3</sub>-HG-KO; *c*, *p* < 0.05 for HG-KO or HG-WT versus VD<sub>3</sub>-HG-WT; *d*, for HG-WT versus HG-KO. HG represents high glucose, and NG represents normal glucose.

Cre± mice to produce FoxO1<sup>fl/fl</sup> Cre<sup>+/-</sup> mice, which were subsequently crossed with FoxO1<sup>fl/fl</sup> mice. FoxO1<sup>fl/fl</sup> mice and their WT littermates were used for the following experiments. All animal care and experiments were conducted in accordance with international standards on animal welfare and approved by the Animal Research Committee of Sichuan University (Chengdu, China). Mouse DM models were established using repeated low doses of (40 mg/kg) streptozotocin (Sigma) via intraperitoneal injection (59). One week after the final injection, mice with a blood glucose > 150 mg/dl higher than control mice were validated for further study.

Forty mice in total were divided into following five groups: normal WT mice (WT); 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated diabetic FoxO1<sup>fl/fl</sup> mice (VD<sub>3</sub>-DB-KO); 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated diabetic WT mice (VD<sub>3</sub>-DB-WT); untreated diabetic FoxO1<sup>fl/fl</sup> mice (DB-KO); and untreated diabetic WT mice (DB-WT). The implant surgery has been previously described (60). 1,25(OH)<sub>2</sub>D<sub>3</sub> was dissolved in propylene glycol and alcohol (4:1) and given at a dose of 5 μg/kg via intraperitoneal injection every other day for 4 weeks. The mice were then sacrificed 2 months post-operation. Serum and femurs with implants were collected for further study.

#### Cell culture and identification

Calvaria from 3-day-old mice were dissected aseptically and placed in tissue culture dish containing PBS and 5× penicillin and streptomycin (pH 7.4). After stripping off the periosteum,

the calvaria were cut into 0.5 × 0.5-mm<sup>2</sup> pieces with scissors and removed into a culture flask containing 5 ml of αMEM supplemented with 10% FBS (Gibco), 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate. The inverted flask was placed in incubator and turned over 2 h later. The calvaria were routinely incubated at 37 °C in 5% CO<sub>2</sub>. Medium was replaced every 3 days until cells were subconfluent. OBs were identified via alizarin red staining and osteocalcin (OCN) immunofluorescence before being used in the following experiments.

OBs in their third passage were detached and reseeded. After a 2-day culture to subconfluence in αMEM containing 10% FBS and 22 mmol/liter glucose, cells from FoxO1<sup>fl/fl</sup> mice and WT mice were respectively trypsinized, reseeded, and treated as follows: normal glucose (5.5 mmol/liter)-treated WT OBs (WT), high glucose (22 mmol/liter), and 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> mol/liter; Sigma)-treated FoxO1<sup>fl/fl</sup> OBs (VD<sub>3</sub>-HG-KO), high glucose-treated and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated WT OBs (VD<sub>3</sub>-HG-WT), high glucose-treated FoxO1<sup>fl/fl</sup> OBs (HG-KO), and high glucose-treated WT OBs (HG-WT).

#### Cell viability and differentiation assay

For viability assay, the cells were seeded at a density of 5 × 10<sup>3</sup>/well in 96-well plates and allowed to adhere for 24 h with αMEM containing 10% FBS. Serum-free medium was applied for another 24-h culture before cells were treated in different conditions described previously. Cell Counting Kit-8 (CCK8, Dojindo, Japan) was employed to detect cell proliferation at 24, 48, and 72 h, respectively. Cell viability was measured by absorbance at 450 nm using a microplate reader SpectroMAX spectrophotometer (NanoDrop). For osteogenic differentiation, alizarin red (Sigma–Aldrich) staining was conducted. Briefly, after a 3-week osteogenic induction with mineralized solution (10 mM β-glycerophosphate, 50 mg/ml ascorbic acid, and 10 nM dexamethasone), medium in a 6-well plates was removed, and cells were fixed with 4% paraformaldehyde for 30 min. The induced deposition was stained using 1% alizarin red S solution at pH 4.2 for 1 h at room temperature. Mineralization of nodules were dissolved with 10% cetylpyridinium chloride, and the absorbance was examined at 562 nm. For ALP assay, cells were respectively harvested at time point of 4 and 8 days and lysed according to four standard freeze-thaw cycles and ultrasonication. Alkaline phosphatase assay kit (Beyotime) was used to evaluate the absorbance at 405 nm of *p*-nitrophenyl, which was a hydrolysate of *p*-nitrophenyl phosphate catalyzed by ALP. The same samples were used to measure total protein levels by the BCA protein assay kit (Beyotime) following the manufacturer’s instructions. The results were expressed in nmol/min/mg protein of produced *p*-nitrophenol (61).

#### Real-time PCR and Western blot analysis

Total RNA from each sample was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized from 1-μg purified RNA templates using PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Shiga, Japan). The expression of ALP, COL-1, OCN, and OPN at 4 and 8 days of culture in different treatment was subsequently quantified with ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) and SYBR Premix Ex Taq



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**Table 1**  
Primer sequences for RT-qPCR

Gene (mouse)	Forward (5' → 3')	Reverse (5' → 3')	Accession no.
FoxO1 <sup>fl</sup>	ACCACTCTGGACGGCATACT	TGAGTCTGGGGCTAGTTTGA	
BGLAP-cre	CAATAGCCCTGGCAGATTC	TGATACAAGGGACATCTTCC	
ALP	AACCCAGACACAAGCATTC	GCCTTTGAGGTTTGTGGTCA	NM_007431.3
COL-1	GATGACGTGCAATGCAATGAA	CCCTCGACTCCTACATCTTCTGA	NM_007742.4
OCN	GCGCTCTGTCTCTCTGACCT	ACCTTATTGCCCTCCTGCTT	NM_031368.5
OPN	CCCGGTGAAAGTGACTGATT	TTCTTCAGAGGACACAGCATTC	NM_009263.3
GAPDH	AAGCCGGGGCCCACTTGAA	GGACTGTGGTCATGAGCCCTTCCA	NM_008084.3

(Takara, Japan). Primers of ALP, COL-1, OCN, OPN, and GAPDH are listed in Table 1. The relative expression levels of the target genes were normalized to the expression of endogenous housekeeping gene GAPDH using 2<sup>-ΔΔCt</sup> method.

Cell protein was obtained using KeyGEN whole cell lysis assay (KeyGEN) as per the manufacturer's instructions. 50 μg of protein extracts were separated by 10% SDS-PAGE gels, transferred onto a PVDF membrane (Millipore Corp., Bedford, MA), and then incubated at 4 °C overnight with rabbit polyclonal anti-VDR (1:100, Abcam), rabbit polyclonal anti-pAkt (1:500, Cell Signaling Technology), rabbit monoclonal anti-Akt (1:500, Cell Signaling Technology), rabbit monoclonal anti-pFoxO1 (1:100, Santa Cruz), rabbit polyclonal anti-FoxO1 (1:100, Santa Cruz), and mouse polyclonal anti-β-actin (1:1000, Cell Signaling Technology), respectively. After the successful binding of HRP-conjugated secondary antibody (1:1000) to primary antibodies, immunoreactive bands were detected using an ECL kit (Millipore, Billerica, MA). The density of the bands were measured by Quantity One (Bio-Rad).

## ELISA

To detect the concentration of tOCN and uOCN, ELISA (Abcam) was conducted according to the manufacturer's instructions. Blood samples and culture supernatants were collected 2 months and 8 days after 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment, respectively. For quantification of uncarboxylated osteocalcin, both serum and supernatant samples were incubated with hydroxyapatite (calcium phosphate tribasic; Sigma) for 1 h. The supernatant containing the uOC was removed and analyzed by the ELISA kit. The percentage of uOC (%uOC) was calculated as the ratio of unadsorbed tOC (*i.e.* the amount remaining in the supernatant fluid to total osteocalcin multiplied by 100) (62). The insulin (Merckodia, Sweden) level and serum value of 25(OH)D<sub>3</sub> (R&D System) were measured by ELISA following the manufacturer's instructions.

## Immunofluorescence and immunohistochemistry

OBs in different groups were seeded on coverslips until confluent. After 4% paraformaldehyde fixing for 20 min and 2% Triton X-100 permeabilizing for 5 min, the cells were then blocked with 5% bovine serum albumin (Sigma) for 30 min and incubated with primary antibodies of VDR (1:50, Santa Cruz Biotechnology), p-Akt (1:50, Cell Signaling Technology), and FoxO1 (1:50, Santa Cruz Biotechnology) respectively at 4 °C overnight, whereas negative control was incubated with PBS. Samples were subsequently incubated with fluorescein isothiocyanate-conjugated secondary antibodies (1:50; Zsjiq Bio Co., Beijing, China) for 1 h. The nuclei were further stained with

DAPI (1:1000; Beyotime, Shanghai, China). The cells were observed using a fluorescent microscopy (Olympus, Tokyo, Japan).

For histology and immunohistochemistry, femurs were harvested 2 months postoperatively and maintained in 4% neutral paraformaldehyde for 2 days. Bones were washed with running water for 12 h before being subjected to decalcification in 10% EDTA-buffered solution for 4 weeks. The samples were then dehydrated using graded ethanol, vitrified by dimethylbenzene, and embedded in paraffin. 5-μm longitudinal sections were obtained. Bones from different groups were stained with hematoxylin and eosin. Additionally, bone sections were immunostained for VDR (Abcam), p-Akt (Cell Signaling Technology), and FoxO1 (Santa Cruz) using rabbit anti-VDR monoclonal antibody, rabbit anti-pAkt monoclonal antibody, and rabbit anti-FoxO1 polyclonal antibody, respectively. All the immunostained sections were quenched in 3% H<sub>2</sub>O<sub>2</sub> for 5 min. Antigens were retrieved in boiling citrate buffer (pH 6.0) for 8 min, three times. After being blocked in 5% goat serum for 30 min to avoid nonspecific staining, sections were incubated with primary antibodies overnight at 4 °C. Specimens were rewarmed at room temperature for 30 min before secondary antibodies were applied to combine primary antibodies. Sections were finally developed using 3,3'-diaminobenzidine (63) and counterstained with hematoxylin.

## Statistical analysis

Statistical analysis was conducted by SPSS 17.0 (SPSS, Inc., Chicago, IL). The results are given as means ± S.D. The data were analyzed using one-way analysis of variance followed by Newman-Keuls Student's *t* test. A significant difference was assumed at *p* < 0.05 (without specific explanation: *a*, *p* < 0.05 for WT *versus* others; *b*, *p* < 0.05 for VD<sub>3</sub>-HG-WT or HG-KO or HG-WT *versus* VD<sub>3</sub>-HG-KO; *c*, *p* < 0.05 for HG-KO or HG-WT *versus* VD<sub>3</sub>-HG-WT; and *d*, for HG-WT *versus* HG-KO).

*Author contributions*—Y. W. and P. G. designed the research. Y. X. and Y. Z. performed the *in vitro* and *in vivo* experiments and collected the data; N. X., Y. Y., and Q. Z. assisted with animal study; and Y. X. and Y. Z. analyzed the data and wrote the paper. All the authors read and approved the final version of the manuscript.

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