Basic fibroblast growth factor inhibits aortic valvular interstitial cells calcification via Notch1 pathway

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ABSTRACT

Calcific aortic valve disease (CAVD) is an active pathological process mediated by abnormal activation and transdifferentiation of valvular interstitial cells (VICs). The present study aims to investigate the function and underlying mechanism of the basic fibroblast growth factor (BFGF) on osteogenic differentiation of VICs. Porcine VICs cultured with osteogenic induction medium are supplemented with or without BFGF. Morphology of VICs is identified by fluorescein isothiocyanate-labeled phalloidin, the cell viability is assessed by the cell counting kit-8 method, and protein and mRNA expression level of osteogenic differentiation markers, including Runx2, osteopontin, and Sp7, are verified by western blot analysis and quantitative real-time PCR, respectively. RNA sequencing is used to identify changes in gene profiles. Alizarin Red S staining is used to measure calcium deposition. The results demonstrate that the content of calcium deposition and the expression level of osteogenic markers are downregulated by supplementing BFGF. Notch1 signaling pathway is extracted as a candidate target after bioinformatics analysis by RNA sequencing. The transfection of si-Notch1 abolishes the calcification inhibitory effect of BFGF. Taken together, our findings shed the light on the mechanism and potential therapeutics of BFGF for CAVD.

Significance of this study

What is already known about this subject?

- Calcific aortic valve disease (CAVD) was an active pathological process mediated by valvular interstitial cells (VICs) with the characteristics of aortic valve leaflet fibrosis and calcification.
- Basic fibroblast growth factor (BFGF) effectively blocked the activation of VICs to myofibroblasts, thereby inhibiting fibrosis of aortic valve.
- The abnormal activation and transdifferentiation of VICs were implicated in CAVD process.

What are the new findings?

- BFGF inhibits calcium deposition of VICs in vitro.
- BFGF attenuates osteogenic differentiation of VICs.
- BFGF inhibits osteogenic differentiation of VICs by promoting Notch1 pathway.

How might these results change the focus of research or clinical practice?

- BFGF might provide an alternative pathway to treat CAVD.

INTRODUCTION

Previous studies have confirmed that calcific aortic valve disease (CAVD) is an active pathological process mediated by valvular interstitial cells (VICs) with the characteristics of aortic valve leaflet fibrosis and calcification, leading to hemodynamic disorders, and ultimately cardiac failure. Aortic VICs are a cluster of dynamic interstitial cells with different phenotypes. Quiescent VICs are activated into myofibroblasts or transformed into osteoblastic phenotypes in CAVD.[1–3] The abnormal activation and transdifferentiation of VICs under pathological conditions lead to the accumulation of an unbalanced extracellular matrix and the deposition of calcified nodules in aortic valve leaflets. Patients with symptomatic CAVD require surgical intervention, and there is currently no effective pharmaceutical treatment that can terminate or reverse CAVD. Therefore, an in-depth understanding of pharmaceutical therapy and its underlying mechanisms is of great significance for the treatment of CAVD.

Basic fibroblast growth factor (BFGF or FGF-2) could regulate a variety of biological functions through binding fibroblast growth factor receptor (FGFR), including cell proliferation, morphogenesis, and apoptosis.[4 5] FGFR belongs to the family of receptor tyrosine kinases and its activation could induce a variety of intracellular signaling pathways. Previous studies have shown that the fibroblast growth factor (FGF) family plays an important role in maintaining circulation system homeostasis and is involved in the progression of various cardiovascular diseases. BFGF is reported to promote angiogenesis and prevent ischemia-reperfusion injury after myocardial infarction in animal model.[6–10] Previous studies have also verified that BFGF effectively blocked the activation of

VICs to myofibroblasts mediated by transforming growth factor β1 (TGF-β1), thereby inhibiting fibrosis of aortic valve.[11]

In view of the potential protective effect of BFGF on the cardiovascular system, its effect on the phenotype and calcification deposition of VICs is still unclear. In this study, we aim to study the function of BFGF in VICs and explore its possible mechanism.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, and penicillin-streptomycin were purchased from Gibco. Type II collagenase and Western Blotting enhanced chemiluminescence (ECL) kit were purchased from Thermo Scientific. PrimeScript RT reagent Kit and TB Green PCR kit were purchased from Takara. BCA kit and SDS lysis buffer were purchased from Beyotime. Antibody against α-smooth muscle actin (α-SMA) (BF9212, 1:1000) was purchased from Affinity, OPN (ab69498, 1:1000) and Sp7 (ab227820, 1:1000) were from Abcam, Runx2 (#12556, 1:500) was from CST, GAPDH (60004-1-Ig, 1:5000) was from Proteintech, Notch1 (GB111690, 1:500) and BMP-2 (GB11252, 1:1000) were from Servicebio, Wuhan.

VIC isolation and culture

Porcine aortic VICs were harvested and isolated from pigs aged 4–5 months (Wufeng, Shanghai). The porcine hearts were collected in an icebox after sacrifice and immediately taken back to the laboratory. Aortic valve leaflets were cut and immersed in precooled phosphate-buffered saline (PBS) solution. The valve leaflets were digested in 2 mg/mL type II collagenase for 15 min and the valve endothelial cells on the surface were gently scraped off with a sterile cotton swab. The valve leaflets were cut into pieces and then digested in type II collagenase for another 2 hours. Undigested tissue was removed by a filtrating screen. The harvested VICs were collected by centrifugation and then seeded on culture dish in DMEM containing 10% fetal bovine serum, and the medium was changed every 2–3 days. The VICs isolation and immunophenotype identification were reported previously.[12] The VICs at passages 3–5 were used for experiment. The osteogenic induction model of VICs in vitro was achieved by culturing with complete medium containing 2 mmol/L sodium dihydrogen phosphate, 50 μg/mL ascorbic acid, and 10^{-7}mol/L insulin.

Fluorescein isothiocyanate-labeled phalloidin staining

The VICs were fixed by 4% paraformaldehyde for 15 min at room temperature. Then, the VICs were incubated with fluorescein isothiocyanate-labeled phalloidin (Servicebio) for 2 hours after penetration with 0.1% Triton X-100. At last, the nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). The morphology of VICs was observed under a fluorescence microscope.

Cell proliferation activity analysis

The VICs were preseeded on a 96-well plate 12 hours in advance with a confluence of 50% before adding BFGF. The cell proliferation activity was measured by cell counting kit-8 (CCK-8) kit (Dojindo, Japan) under the guidance of manufacturer. In brief, the VICs were incubated for 2 hours by a combination of 90 μL complete medium and 10 μL CCK-8 reagent to each well, and then the absorbance at 450 nm was measured by Multiscan Spectrum (Biotek, USA).

Alizarin Red S staining

The VICs were fixed by 75% ethanol for 15 min at room temperature after 7 days of osteogenic induction, and then the VICs were stained with Alizarin Red S Kit (Servicebio) for 5 min after being rinsed 3 times with deionized water. Subsequently, the non-specific staining was removed by 95% ethanol. At last, the staining was captured by camera and microscope. As for the semi-quantitative analysis of Alizarin Red S staining, 500 μL of 10% acetic acid solution was used to dissolve the calcium deposition each well. The absorbance at 450 nm was measured by Multiscan Spectrum.

mRNA sequencing

mRNA sequencing (RNA-seq) was used to quantitatively analyze the changes in the cellular mRNA profile among different treatments. In short, the RNA extracted from different treatments was sent to BGI Gene (Shanghai, China) for RNA sequencing. 'R language' (V.3.5.1) was used to further analyze the sequencing results, including differentially expressed genes (DEGs), Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Polymerase chain reaction

The VICs were seeded in 6-well plate, and the total RNA was extracted 48 hours after each treatment; 1 mL Trizol was added to each well to lyse the VICs after being washed 3 times with PBS. The extraction procedure was under the guidance of manufacturer's instruction. At last, the RNA precipitation was dissolved in 20 μL enzyme-free water. Complementary DNA was synthesized by a PrimeScript RT reagent Kit. Finally, PCR was performed by TB green PCR kit. The primer sequences are shown in table 1. GAPDH was set as reference, and the untreated VICs group mRNA expression was normalized as 1. All results were calculated by $2^{-\triangle\triangle ct}$ method.

Western blot analysis

The VICs were seeded in a 6-well plate, and the total protein was lysed 72 hours after each treatment; 150 μL SDS lysis

Table 1 Primer sequence

Gene	Primer sequence
Sus-Runx2-F	CAGACCAGCAGCACTCCATA
Sus-Runx2-R	AACGCCATCGTTCTGGTTAG
Sus-OPN-F	CTTGGACAGCCAAGAGAAGG
Sus-OPN-R	TGGCTGACTTTGGGATTTTC
Sus-Sp7-F	ACCAATGGGCTCCTGTCAC
Sus-Sp7-R	CACTGGGCAGACAGTCAGAA
Sus-α-SMA-F	GCCCTGGACTTTGAAAATGA
Sus-α-SMA-R	CCCCAGAGAGGACGTTGTTA
Sus-GAPDH-F	GTCGGTTGTGGATCTGACCT
Sus-GAPDH-R	AGCTTGACGAAGTGGTCGTT

α-SMA, α-smooth muscle actin.

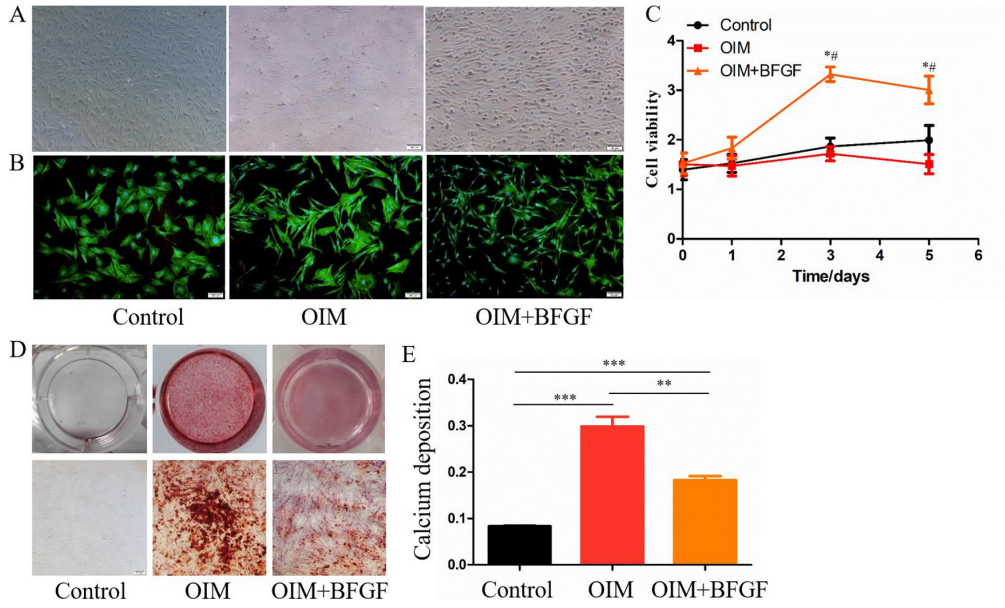

Figure 1 Cell viability and calcium deposition of VICs with treatment of BFGF. (A) Cell morphology of VICs cultured in OIM with or without supplement of 50 ng/mL BFGF, VICs cultured with DMEM were set as control. Scale bar=100 µm. (B) Cell morphology observed by fluorescein isothiocyanate-labeled phalloidin. Scale bar=100 µm. (C) Cell viability of VICs with different conditioned culturing identified by Cell Counting Kit-8: control (DMEM), OIM, and OIM+BFGF. *P<0.05 compared with control group. #P<0.05 compared with OIM group. (D) Alizarin Red S staining of VICs with different conditioned culturing. (E) Semi-quantitative analysis of calcium deposition of VICs with different conditioned culturing. **P<0.01, ***p<0.001. BFGF, basic fibroblast growth factor; DMEM, Dulbecco's Modified Eagle Medium; OIM, osteogenic induction medium; VICs, valvular interstitial cells.

buffer supplemented with 1:100 protease and phosphorylase inhibitors was added to each well. The protein concentration was measured and standardized by the BCA kit. The target protein was separated by 10% PAGE and then transferred to the polyvinylidene fluoride (PVDF) membrane. The primary antibody was incubated at 4° overnight at a recommended concentration after the block procedure with skimmed milk. Subsequently, corresponding secondary antibody was incubated for 1 hour at room temperature. Finally, the membrane was exposed to film by ECL kit.

Statistical analysis

All data were expressed as mean±SD. The two groups were compared using Student's t-test, and the analysis of variance (ANOVA) was used to test the differences between groups. Bonferroni's test was performed for multiple comparative analyses between two independent groups after ANOVA. All experiments were repeated at least 3 times, and $p<0.05$ indicated that the difference was statistically significant.

RESULTS

BFGF inhibits calcium deposition of VICs in vitro

At first, in order to investigate the effect of BFGF on VICs, 50 ng/mL BFGF was supplemented into osteogenic induction medium (OIM). The morphology of VICs gradually transformed into a long spindle shape after the introduction of BFGF (figure 1A,B). Then, the cell proliferation activity of VICs was tested by CCK-8 kit. The proliferation activity of the BFGF group was significantly higher than that of the control after 3 days of continuous culture (figure 1C). At last, Alizarin Red S staining was used to detect calcium deposition after 7 days of continuous culture of VICs. Of note, compared with the control group, Alizarin Red S staining was positive in the OIM and BFGF groups (figure 1D). The semi-quantitative analysis results of calcium deposition showed that the addition of BFGF significantly inhibited the calcium deposition of VICs (figure 1E).

BFGF attenuates osteogenic differentiation of VICs in vitro

We first detected the expression of osteogenic differentiation markers of VICs, including osteopontin, runt-related transcription factor 2 (Runx2), and Sp7, by western blot analysis. The results showed that osteogenic differentiation proteins were significantly upregulated after 72 hours of continuous culture of VICs. In contrast, their expression levels were decreased when supplemented with BFGF (figure 2A–D). Meanwhile, VICs predominantly exhibit a fibroblast phenotype under quiescent conditions. It was reported that the process of osteogenic differentiation of VICs was accompanied by the activation of VICs into myofibroblasts. The expression of α-SMA was significantly upregulated after the addition of OIM, and its upregulation was abolished after supplementation with BFGF (figure 2E). In addition, we further confirmed that the mRNA expression of osteogenic differentiation markers was significantly downregulated after adding BFGF (figure 2F–I). The above results suggest that BFGF may prevent the osteogenic differentiation of VICs.

Bioinformatics analysis

In order to investigate the underlying mechanism of osteogenic differentiation inhibited by BFGF, RNA-sequencing was performed. The gene expression profiles of

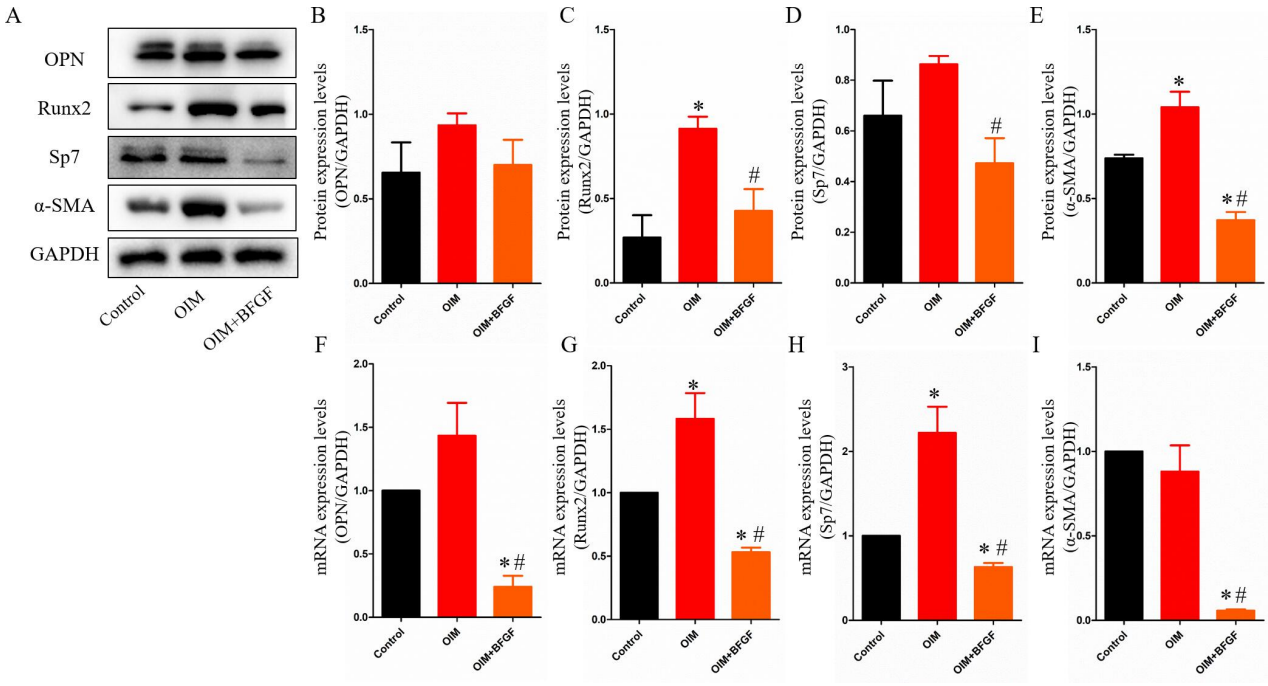

Figure 2 Effect of BFGF on osteogenic marker expression of VICs. (A) Representative western blot analysis photo of osteogenic protein expression. (B–E) Semi-quantitative analysis of osteogenic protein expression level, including OPN, Runx2, Sp7, and α-SMA. *P<0.05 compared with control group. #P<0.05 compared with OIM group. (F–I) The mRNA expression level analysis of aforementioned osteogenic marker. *P<0.05 compared with control group. #P<0.05 compared with OIM group. α-SMA, α-smooth muscle actin; BFGF, basic fibroblast growth factor; OIM, osteogenic induction medium; Runx2, runt-related transcription factor 2; VICs, valvular interstitial cells.

BFGF+OIM and OIM groups were significantly different, including an increase of 1260 and a decrease of 1266 (figure 3A). There were 159 differential gene expressions between OIM and control group, including 63 increased and 96 decreased (figure 3B). Seventy-four DEGs were list for further analysis by using the Venn diagram (figure 3C). According to the gene expression profile, the OIM and the control groups were similar and were classified as the same cluster (figure 3D). Enrichment analysis of KEGG pathway(figure 3E, online supplemental file 1) showed that DEGs have highly enriched Jak-STAT and Notch signaling pathways.

BFGF inhibits osteogenic differentiation of VICs by promoting Notch1 pathway

Among all potential pathways, Notch1 attracted our attention and was further studied. First, the expression of Notch1 was increased after the addition of BFGF, which was identified by western blot analysis. On the contrary, the expression of BMP-2, a downstream gene of Notch1, was decreased significantly (figure 4A–C). Alizarin Red S staining showed that transfection of si-Notch1 abolished the calcification inhibitory effect of BFGF (figure 4D,E). The expression of Notch1 was downregulated and the expression of BMP-2 was upregulated after transfection of si-Notch1 (figure 4F). Taken together, the results suggested that the inhibitory effect of BFGF on the osteogenic differentiation of VICs was partly dependent on Notch1 pathway.

DISCUSSION

Previous studies have shown that CAVD was a pathological process of ectopic calcification deposition. In this study, we, for the first time, found that exogenous BFGF could significantly inhibit the osteogenic differentiation of VICs, suggesting that BFGF may act as a potential candidate of the drug treatment for CAVD. Meanwhile, we verified that its inhibitory effect was achieved by inhibiting Notch1 signaling pathway through bioinformatics analysis, which was further validated by VICs osteogenic differentiation model in vitro. Interestingly, the expression level of BFGF was also significantly increased in atherosclerotic plaques. The introduction of exogenous BFGF inhibited TGF-β signaling and significantly inhibited vascular smooth muscle cell calcification.[13] BFGF was also proved to promote VICs repair.[14] Taken together, we supposed that BFGF might own potential protective effect on the cardiovascular system.

In this study, we reported that the phenotypic transformation of VICs induced by BFGF may be mediated by its accelerative effect on Notch1 signaling pathway. Notch was reported to act as an important role in heart development and disease. Notch was involved in almost all stages of heart development, including fate determination of the heart, the formation of the primitive heart tube, and the morphology of left ventricular outflow tract. It was reported that Notch signaling pathway was involved in early myocardial differentiation, trabecular myocardium formation, and valves and cardiac conduction system formation.[15–17] There are four transmembrane Notch

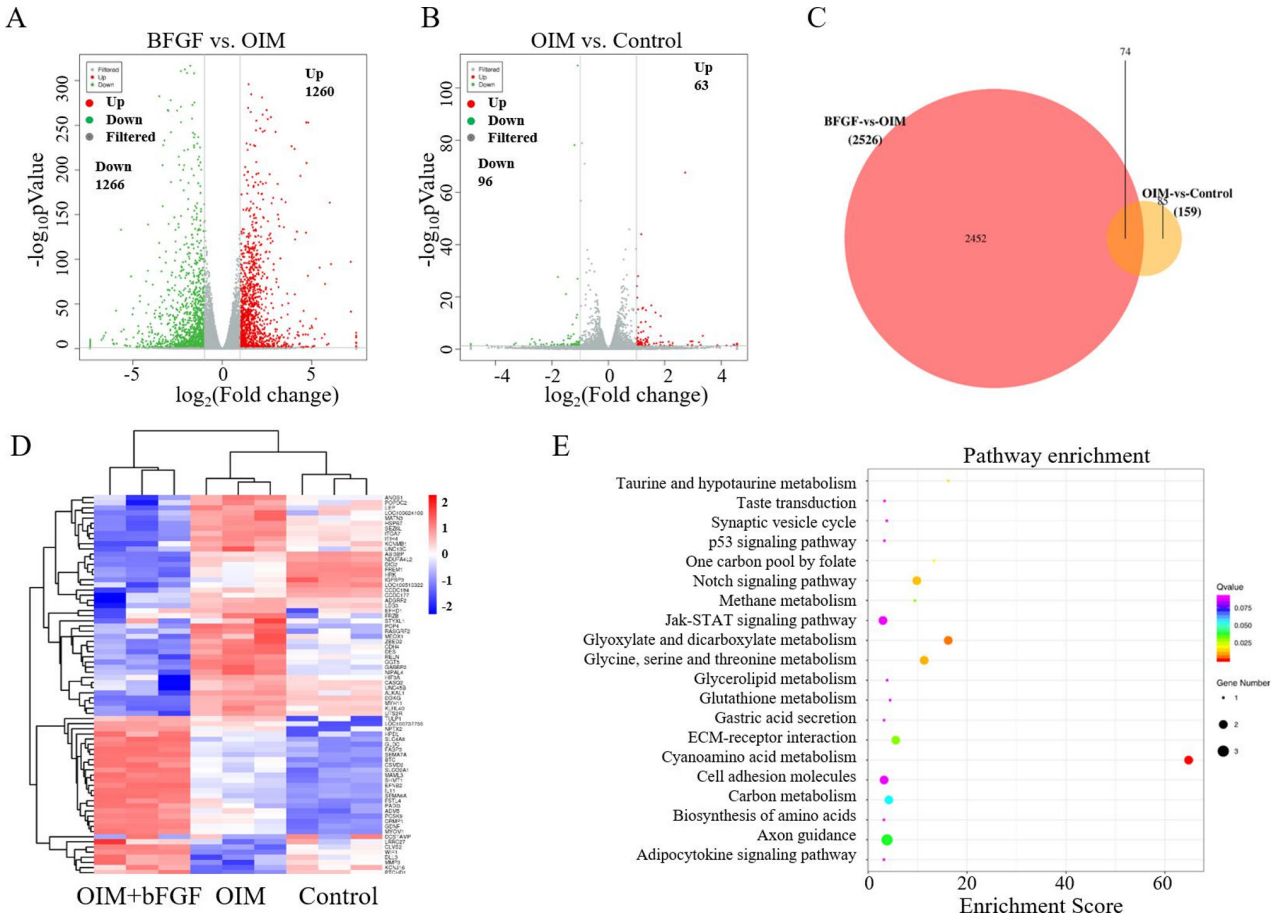

Figure 3 Gene expression profile with RNA-sequencing of VICs with treatment of BFGF. (A and B) Volcano map of DEGs in OIM+BFGF versus OIM and OIM versus control. (C) Venn interaction of DEGs of OIM+BFGF versus OIM and OIM versus control. (D) Heatmap for common DEGs with group clusters. (E) KEGG pathway enrichment, bubble size indicates gene counts matched the pathway enrichment. BFGF, basic fibroblast growth factor; DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; OIM, osteogenic induction medium; VICs, valvular interstitial cells.

receptors in mammals, namely Notch 1–4. Notch1 was reported to participate in endocardial-mesenchymal transition, which was essential for the formation of aortic and pulmonary valves. In addition, Notch1 was demonstrated to inhibit valve calcification by regulating the expression of BMP-2 and inhibiting the expression of pro-inflammatory and osteogenic genes.[18 19]

Notch cross-talks reiteratively with Wnt, BMP-2, and TGF-β during valve development and valve disease.[20 21] Hyun *et al* found that BFGF eliminated the osteogenic effect of BMP-2 on bone formation of human periodontal ligament stem cells, indicating that the function of BFGF was BMP-2 pathway-dependent.[22] Inhibition of Notch1 was also demonstrated to promote the osteogenesis of VICs by targeting BMP-2 pathway.[23] Nigam and Srivastava have demonstrated that heterozygous Notch1 knockout mice were prone to CAVD when compared with wild-type mice. Knockdown of Notch1 expression in goat aortic VICs enhanced the expression of the phenotype of osteoblasts, including BMP-2 and osteopontin. Additionally, the expression of BMP-2 was essential for the regulation of osteogenic differentiation of VICs by Notch1, which was identified by rescue experiment.[23] The activity of alkaline phosphatase was reduced after the addition of 10 ng/mL BFGF in osteogenic differentiation model of mesenchymal stem cells isolated from human exfoliated deciduous teeth, and the inhibitory effect of BFGF on calcification was inhibited by FGFR antagonists.[24]

The imbalance of classic Notch-Wnt pathway has been confirmed to participate in CAVD process. The expression disorder of Notch1 may also be involved in the pathological process of bicuspid aortic valve, which was confirmed to be a high-risk factor of CAVD.[25] Functional mutation of Notch1 increased the tendency of valve calcification through upregulating the expression of Runx2.[26 27] Runx2 was a key regulatory factor of osteoblast differentiation, which regulated the expression of osteopontin, osteonectin, and other osteogenic targets. The mechanism involved in Notch1 has not yet been elucidated, but the cell fate and osteogenic transformation of VICs were controlled by the Notch1 pathway to some extent.

BFGF was reported to inhibit calcium deposition of VICs by inhibiting the activation of VICs in addition to preventing osteogenic differentiation of VICs by targeting Notch1 pathway. Previous studies have confirmed that a fraction of VICs in normal aortic valve owns the phenotype of myofibroblasts with positive expression of α-SMA.[2] Abnormal activation of VICs to myofibroblasts

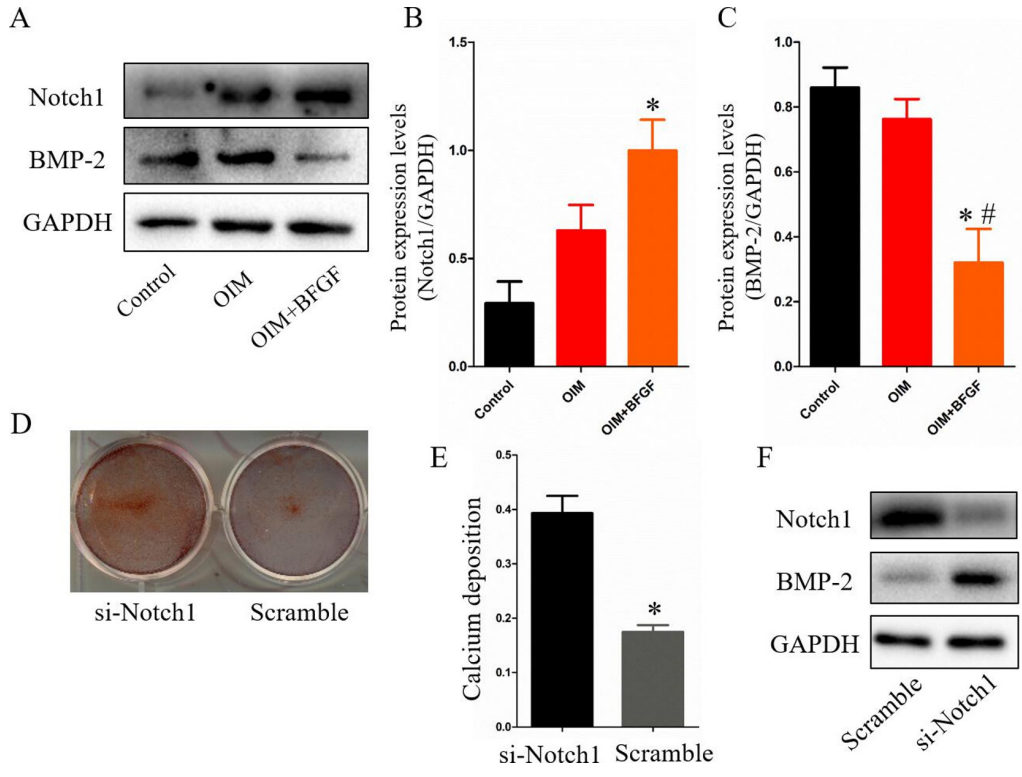

Figure 4 BFGF inhibits calcification of VICs via Notch1 signaling pathway. (A) Representative western blot analysis photo of Notch1 and BMP-2. (B and C) Semi-quantitative analysis of protein expression level of Notch1 and BMP-2. (D) Alizarin Red S staining of VICs after transfection of si-Notch1. (E) Semi-quantitative analysis of calcium deposition of VICs.*(F) Protein expression level of Notch1 and BMP-2 after transfection of si-Notch1. *P<0.05 compared to control group. #P<0.05 compared to OIM group. BMP-2, bone morphogenetic protein-2; BFGF, basic fibroblast growth factor; OIM, osteogenic induction medium VICs, valvular interstitial cells.

mediated by TGF-β signaling pathway may lead to valve calcification. Previous studies have suggested that BFGF prevents VICs from calcification by maintaining VICs in a quiescent condition.[11 28] BFGF was reported to reduce activation of the myofibroblast phenotype, including reduce α-SMA expression, collagen deposition, fibrotic contraction, and calcific nodule formation in aortic VICs.[5 11 29 30] One possible mechanism of inhibitory effect of myofibroblasts activation was that BFGF repressed the nuclear translocation of Smad transcription signals.[5] On the other hand, we found that the addition of BFGF accelerated the proliferation of VICs, which was identified to the results of dental pulp stem cells.[31 32] And the activation of the ERK pathway promoted the proliferation of dental pulp stem cells.

At last, the FGF superfamily has been proven to be widely involved in the pathological process of cardiovascular diseases. For example, FGF23 was proved to participate in calcium and phosphorus metabolism in patients with chronic kidney disease. A high level of FGF23 in patients with chronic kidney disease inhibited the classic osteogenic pathway Wnt-β-catenin and led to osteoporosis. An elevated level of FGF23 was positively correlated with cardiovascular mortality in patients with chronic kidney disease.[33] FGF21 was proved to inhibit vascular calcification by regulating apoptosis and endoplasmic reticulum stress of vascular smooth muscle cells.[34 35] In the study, we confirmed that BFGF suspended osteogenic differentiation of VICs in vitro, suggesting that BFGF might have the potential to treat CAVD. However, the challenge of metabolic disorders in bone tissue or other organs induced by BFGF requires further exploration.

Contributors HL and LX conceived the ideas. GY and LN designed the experiments. GY performed the experiments. LN analyzed the data. XQ and FX provided critical materials. LN and GY wrote the manuscript. HL and LX supervised the study. All the authors have read and approved the final version for publication. HL was responsible for the overall content as the guarantor.

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