Bone 123 (2019) 196–203

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Silencing GSK3β instead of DKK1 can inhibit osteogenic differentiation caused by co-exposure to fluoride and arsenic

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ARTICLE INFO

Keywords:
Fluoride
Arsenic
Bone
GSK3β
DKK1

ABSTRACT

Chronic exposure to combined fluoride (F) and arsenic (As) continues to be a major public health problem worldwide, attracting the attention of an increasing number of researchers. While bone is the main target organ of syndrome of endemic arsenic poisoning and fluorosis (SEAF), the specific mechanism and targeted intervention remains uncertain. The first question in this study sought to determine the interaction of F and As on the Wnt signaling pathway and its role in osteogenic differentiation in the SEAF population. As can be seen from the data, with the increase in exposure to F, the content of Wnt signaling inhibitor Dickkopf-related protein 1 (DKK1) gradually decreased, but the expression of glycogen synthase kinase-3β (GSK3β), β-catenin and the osteogenic differentiation indicators pro-collagen I alpha 1 (COL1A1) and bone alkaline phosphatase (BALP) were increased. Next, we grouped the SEAF population according to urinary As and found that As can upregulate the GSK3β, β-catenin level and the bone formation bio-marker BALP in serum. But the experiments did not detect any evidence that As can change the content of DKK1 in serum. To better understand the combined effects of F and As on the Wnt signaling pathway, we performed further interaction analysis. These results suggest that the interaction of F and As can inhibit the GSK3β, β-catenin, COL1A1 and BALP. And DKK1 is mainly manifested by the independent effect of F. To further study the role of DKK1 and GSK3β in fluoride-arsenic pollution combined with osteogenic differentiation, we attempted to silence the DKK1 and GSK3β gene in hFOB 1.19 cells. The results show that F, As alone and in combination exposure can up-regulate GSK catenin transcription and protein expression levels and down-regulate DKK1, and COL1A1 and ALP are significantly increased, after silenced the DKK1. The same results did not appear after silenced the GSK3β. F and As alone and in combination exposure did not reverse the inhibition of GSK3β and β-catenin by GSK3β silencing, and COL1A1 and ALP are significantly decreased. The results indicate that silencing GSK3β instead of DKK1 can inhibit osteogenic differentiation caused by co-exposure to fluoride and arsenic. This study can provide a scientific basis for further understanding the causes of bone formation caused by F and As and the improvement of targeted intervention strategies.

1. Introduction

Both inorganic fluoride (F) and arsenic (As) are ubiquitous in the natural environment. Concurrent chronic poisoning has been referred to as syndrome of endemic arsenic poisoning and fluorosis (SEAF) [1], and it continues to be a major public health problem worldwide, affecting thousands of people [2]. Many studies have reported simple fluorosis and arsenic poisoning; however, very little is known about the combined effect of these two chemicals. An excess of F and As in the environment has been reported in many countries, such as China, India, Japan, Korea, Argentina, Bolivia, Chile, Colombia and Mexico [3]. Drinking water and burning coal are the two sources of SEAF, with the latter type unique to China. The burning coal type of SEAF affects residents living in a specific geographical environment and is caused by excessive intake of F and As through the air or food. Guizhou Province has always been the area most impacted by SEAF in China.

According to reports, combined exposure to F and As can cause damage to bone [4–7], skin [4], brain [4,8,9], liver [4,10], kidney [11,12], heart [4,10], and reproductive development [13]. Bone has been the focus of attention for researchers. Osteoblasts and osteoclasts are the two major cells that maintain bone mass during bone remodelling. Under normal circumstances, osteoblasts and osteoclasts

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https://doi.org/10.1016/j.bone.2019.03.016
Received 13 August 2018; Received in revised form 12 March 2019; Accepted 15 March 2019
Available online 16 March 2019

maintain a certain amount and restrict each other, so that bone formation and bone resorption are in a state of dynamic equilibrium. *In vitro* and *in vivo* studies reported that F has anabolic effect on bone metabolism and stimulates osteoblastic bone formation [14]. However, the effect of As on bone formation has been controversial. Results from an experimental study in population [7] and rats [5,6] have suggested that there is an interaction between F and As on bone formation. Nevertheless, the specific mechanism and targeted intervention of bone formation caused by combining exposure to F and As remain uncertain.

The Wnt signaling pathway is a research hotspot in the pathogenesis and bone metabolism of skeletal system-related diseases in recent years and has become an important target for the treatment of various bone diseases [15–19]. An *in vivo* study has demonstrated that F induced β-catenin mRNA and protein expression in the bones of rats [20]. Other studies reported that decreased Dickkopf-related protein 1 (DKK1) and Sclerostin (SOST) levels, the potent antagonists of Wnt/β-catenin signaling pathway, were found in serum of fluorosis patients and fibroblasts exposed to F [21,22]. Moreover, in primary rat osteoblasts, F promotes osteoblastic differentiation through glycogen synthase kinase-3β (GSK3β)-dependent activation of the Wnt/β-catenin signaling pathway, and the osteogenic differentiation indicators alkaline phosphatase (ALP), pro-collagen I alpha 1 (COL1A1) and osteonectin significantly increased [23]. Interestingly, the positive effect of F on above indicators was abolished by DKK1. These findings indicate that Wnt/β-catenin signaling pathways maybe involved in bone formation caused by F, and DKK1 and GSK3β plays an important role in fluoride-induced bone formation. Very little is known about the effect of As on bone damage through the Wnt signaling pathway. Nevertheless, in the study of colorectal tumours, a few studies [24,25] found that As can promote the activation of the Wnt/β-catenin signaling pathway through oxygen-free radicals and promote the occurrence of colorectal tumours. This suggests that As may affect the activation of the Wnt/β-catenin signaling pathway. Although co-exposure to F and As is known to stimulate bone formation [5–7], the underlying mechanisms are not fully understood. In addition, the key targets of the Wnt signaling pathway for bone toxicity in combination with F and As is unknown. Therefore, more in-depth research is necessary.

In this paper, the changes in gene and protein expression of the classical Wnt signaling pathway and the osteogenic differentiation markers COL1A1, bone alkaline phosphatase (BALP) in each group were compared, in order to study the role of Wnt signaling pathway in osteogenic differentiation caused by combined exposure to F and As. Then, we attempted to silence the DKK1 and GSK3β genes in hFOB1.19 osteoblastic cells, to further study the role of DKK1 and GSK3β in fluoride-arsenic pollution combined with osteogenic differentiation. The study can provide a scientific basis for further understanding the causes of the bone formation caused by co-exposure to F and As and the improvement of targeted intervention strategies.

2. Materials and methods

2.1. Study population and groups

This was a population-based study. The selected investigation site was located in Liuchang Village of Qinzhen City in the Guizhou Province of China. This area is a pollution ward with an excess of F and As in the environment. A F&As-free area in the suburbs of Qingzhen City was chosen as a control site. The F and As content of these areas in the environment are shown in Fig. S1. The eating habits, economic status and nutritional status of the control area were similar to that of the exposure area. Written informed consent was obtained from all participants. The study proposal was reviewed and approved by the Ethical Committee of Guiyang Medical University. We worked with the Guiyang Centre for Disease Control and Prevention and its subordinate units to recruit volunteers. All participants were required to be permanent residents of the local area (Liuchang Village) and were matched for age and sex. The total number of participants was 196. Exclusion criteria included smoking, drinking, pregnancy and recent history of consuming seafood or drugs, which could affect urinary excretion of F and As. The reference values of urinary F and urinary As are provided [7], with double geometric spacing, F was divided into $F_{<1.2}$, $F_{1.2–2.4}$, $F_{>2.4}$ mg/gCr three levels, and As was divided into $As_{<20}$, $As_{20–40}$, $As_{>40}$ μg/gCr three levels. The characteristics of the study participants and number of samples in each group are presented in Tables S1 and S2.

2.2. Sample collection

After obtaining informed consent, fasting venous blood samples and morning urine samples were collected. All participants were instructed on how to avoid contamination. All samples were stored at −20 °C until analysis.

2.3. F, As and creatinine concentration in urine

Each urine sample was divided into three aliquots for the determination of urinary F, As and creatinine. F was determined by the fluorine ion selective electrode method (PXJ-1B digital ion meter), and As was determined by the atomic fluorescence spectrometry method (AF-610D2 chromatography-atomic fluorescence spectrometry). The above methods are the national health industry standard method in China. The concentration of urinary creatinine was determined by the Jaffe reaction method (T6 UV–visible spectrophotometer). All urinary parameters were standardised to the concentration of creatinine in urine.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Two millilitres of non-anticoagulated blood were used to separate serum for the measurement of DKK1, GSK3β, β-catenin and BALP, COL1A1 protein concentrations by the ELISA method (BioTek MQX200 UQuant Microplate Reader, USA) in the serum of participants. Determination of secreted BALP in cells using ELISA method. These biomarkers were determined using ELISA kits from the CUSABIO (DKK1, GSK3β, β-catenin, BALP) and Abcam (COL1A1) Co., Ltd.

2.5. Cell culture, transfection and treatments

The hFOB 1.19 cells, purchased from the American Type Culture Collection (ATCC) cell bank, were cultured in a Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Gibco, Invitrogen, USA), supplemented with 10% foetal bovine serum (FBS) (Gibco, Invitrogen, USA), in a humidified 5% CO_2 atmosphere at 37 °C with the medium being changed every other day. This study used a total of eight cell treatments, including SIRP-DKK1/GSK3β negative control (NC), SIRP-DKK1/GSK3β NC + 0.1 μM $NaAsO_2$, SIRP-DKK1/GSK3β NC + 1 mM NaF, SIRP-DKK1/GSK3β NC + 0.1 μM $NaAsO_2$ + 1 mM NaF, SIRP-DKK1/GSK3β, SIRP-DKK1/GSK3β + 0.1 μM $NaAsO_2$, SIRP-DKK1/GSK3β + 1 mM NaF and SIRP-DKK1/GSK3β + 0.1 μM $NaAsO_2$ + 1 mM NaF. siDKK1 and siGSK3β were designed and synthesised by RIBOBIO Co., Ltd. The siRNA sequences are presented in Table S3. The solutions were diluted to 20 mM with diethyl pyrocarbonate (DEPC) water before use. Logarithmically grown cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and cultured for 24 h. 10× riboFECT™ CP Buffer (V1) was diluted to 1× riboFECT™ CP Buffer (V2) before taking 120 μL of V2, mixing gently with 5 μL of siRNA stock solution (V3), adding 3 μL of riboFECT™ CP reagent (V4), mixing gently by pipetting and incubating for 7 min at room temperature. To the above solution, 1,683 μL of the medium was added to prepare a mixed solution, which was added to a 6-well plate for culturing for 24 h. Then, cells were treated with $NaAsO_2$ (0.1 μM) (Sigma, USA) and or NaF (1 mM) (Sigma, USA) in DMEM containing 1% FBS according to the design of this study. After 24 h of culture, the cells were harvested for subsequent experiments.

2.6. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated, and reverse transcription was performed with a Takara Prime Script ™ II 1st Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan) according to manufacturer's instructions. The levels of mRNA expression were examined using the quantitative PCR method (CFX96, BIO-RAD, USA) and analysed with the Bio-Rad CFX Manager 3.1 (BIO-RAD, USA). The primer sequences are presented in Table S4.

2.7. Western blotting

After treatment for 24 h, the cells were washed twice with cold PBS, and ice-cold RIPA lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to the cells. After the cells were completely lysed, the lysates were transferred to a microcentrifuge tube and centrifuged at 12,000*g* for 15 min at 4 °C. Supernatants containing total protein were harvested. Aliquots, each containing 50 μg of protein, were separated by a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes at 300 mA for 1 h at low temperature. The membranes were blocked in 5% skimmed milk for 2 h. Subsequently, proteins were detected using monoclonal antibodies at 1:200 (DKK1, GSK3β, β-catenin) and 1:500 (phospho-GSK3β, ALP), 1:1000 (COL1A1) dilutions for overnight at 4 °C, and then visualised using anti-mouse or anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase at a 1:8000 dilution for 2 h at room temperature, respectively. A ChemiDoc™ Imaging System (ChemiDoc, BIO-RAD, USA) was used to catch up the specific bands, and the optical density of each band was measured using Image Lab™ software (Image Lab, BIO-RAD, USA). The β-actin was used as an internal control and used to correct for differences in protein loading, to normalize the blot.

2.8. Statistical analysis

Data were analysed using SPSS 13.0 software. A one-factor analysis of variance was used to evaluate the differences between groups, and the least significant difference test was used for *post hoc* subgroup analysis. The interaction between F and As was analysed using a general linear regression model. All data were presented as mean ± standard deviation of at least three independent experiments. Results were considered statistically significant when the *p-value* was < 0.05 and presented in the form of graphs, produced using GraphPad and S-PLUS software.

3. Results

3.1. Wnt signaling pathway is involved in bone damage co-exposure to F and As in Chinese population

3.1.1. The dose-effect relationships between F, As and the Wnt signaling pathway

By observing the changes in protein expression of the Wnt signaling pathway after exposure to different concentrations of F and As, the results are consistent in male and female people that the differences of DKK1, GSK3β and β-catenin between the various F exposed groups was significant. The concentration of GSK3β and β-catenin in $F_{1.2–2.4}$ mg/gCr, $F_{>2.4}$ mg/gCr groups were increased compared to the $F_{<1.2}$ mg/gCr group ($p < 0.05$). In contrast, the concentration of DKK1 in $F_{1.2–2.4}$ mg/gCr, $F_{>2.4}$ mg/gCr groups were lower than the $F_{<1.2}$ mg/gCr group ($p < 0.05$). Compared with the $F_{1.2–2.4}$ mg/gCr group, the concentration of GSK3β and β-catenin in the $F_{>2.4}$ mg/gCr group were increased, but the concentration of DKK1 in $F_{1.2–2.4}$ mg/gCr was lower than $F_{>2.4}$ mg/gCr group ($p < 0.05$). These results are shown in Fig. 1A. Fig. 1B shows that the concentration of GSK3β and β-catenin in the $As_{20–40}$ μg/gCr and $As_{>40}$ μg/gCr groups were higher than the $As_{<20}$ μg/gCr group ($p < 0.05$). There was no significant difference between the $As_{20–40}$ μg/gCr and $As_{>40}$ μg/gCr groups in the concentration of GSK3β and β-catenin, and the concentration of DKK1 was not different in the different As groups (p *all* > 0.05).

3.1.2. The interactions between F and As on the Wnt signaling pathway

Based on the concentration of F and As, F below 1.2 mg/gCr and As < 20 μg/gCr for the control group. It is a single exposure to As if the group with $F_{<1.2}$ mg/gCr $As_{20–40}$ μg/gCr (As_{low}), $F_{<1.2}$ mg/gCr $As_{>40}$ μg/gCr (As_{high}) groups. Meanwhile, the group with $As_{<20}$ μg/gCr $F_{1.2–2.4}$ mg/gCr (F_{low}) and $As_{<20}$ μg/gCr $F_{>2.4}$ mg/gCr (F_{high}) as the single exposure to F. The interaction groups included $F_{1.2–2.4}$ mg/gCr $As_{20–40}$ μg/gCr ($F_{low}As_{low}$), $F_{1.2–2.4}$ mg/gCr $As_{>40}$ μg/gCr ($F_{low}As_{high}$) and $F_{>2.4}$ mg/gCr $As_{20–40}$ μg/gCr ($F_{high}As_{low}$) group, and the $F_{>2.4}$ mg/gCr $As_{>40}$ μg/gCr ($F_{high}As_{high}$) group had no sample in this study.

As shown in Fig. 1C and F, F alone can affect the concentration of DKK1 ($p < 0.05$), but not As alone ($p > 0.05$). F and As have no interaction with DKK1 ($p > 0.05$). DKK1 in the $F_{low}As_{low}$, $F_{low}As_{high}$ and $F_{high}As_{low}$ groups was lower than the single As group (p *all* < 0.05), but no significant differences were found in the single F group ($p > 0.05$).

It can be seen from the data in Fig. 1D and G that both F and As can affect the concentration of GSK3β, the difference in the interaction between F and As on GSK3β was statistically significant (p *all* < 0.05). Compared with the single F or As group, the concentration of GSK3β in $F_{low}As_{low}$, $F_{low}As_{high}$ were increased, and the GSK3β in $F_{high}As_{low}$ group was higher than the As_{low} group (p *all* < 0.05), but there was no significant difference between $F_{high}As_{low}$ and F_{high} group ($p > 0.05$).

Fig. 1E and H show that single F, As and its combination can both affect the concentration of β-catenin (p *all* < 0.05). The content of β-catenin in $F_{low}As_{low}$, $F_{low}As_{high}$ and $F_{high}As_{low}$ were higher than F or As alone (p *all* < 0.05).

3.1.3. The influences of F and As on effective biomarker COL1A1 and BALP

COL1A1 and BALP are important biochemical indicator for osteogenic differentiation in bone metabolism. Fig. 1I shows that the concentration of COL1A1 and BALP in $F_{1.2–2.4}$ mg/gCr, $F_{>2.4}$ mg/gCr groups were increased compared to the $F_{<1.2}$ mg/gCr group, $F_{>2.4}$ mg/gCr groups is higher than $F_{1.2–2.4}$ mg/gCr group (p *all* < 0.05). Moreover, Fig. 1J shows that the concentration of COL1A1 and BALP in the $As_{20–40}$μg/gCr, $As_{>40}$ μg/gCr groups was significantly increased compared to the $As_{<20}$ μg/gCr group (p *all* < 0.05), but there was no significant difference between the $As_{20–40}$ μg/gCr and $As_{>40}$ μg/gCr groups in the concentration of COL1A1 and BALP (p *all* > 0.05). Further interaction analysis (Fig. 1K, L, M and N) showed that F alone, As and its combination can both affect the concentration of COL1A1 and BALP (p *all* < 0.05). The concentration of COL1A1 and BALP in $F_{low}As_{low}$, $F_{low}As_{high}$ and $F_{high}As_{low}$ were higher than F or As alone (p *all* < 0.05).

3.2. Silencing DKK1 can promote osteogenic differentiation by increasing the activation of Wnt signaling caused by F, As and their combination

To study the effects of F and As on the osteogenic differentiation of hFOB 1.19 cells, sodium fluoride and sodium arsenite solutions were used at different concentrations and various times to obtain the best dose (1 mM NaF and 0.1 μM $NaAsO_2$) and time (24 h), according to the cell viability of hFOB 1.19 cells. In order to understand the key target of the Wnt signaling pathway for osteoblastic differentiation in combination with F and As, siRNA was used to silence the DKK1 gene. Fig. 2A show that the DKK1 transcriptional expression in SIRP-DKK1 group was significantly lower than the SIRP-DKK1 NC group ($p < 0.05$). Compared with SIRP-DKK1 NC group, DKK1 protein expression level decreased by 50% (Fig. 2D). After silencing DKK1, co-exposure to F and As or exposure to F alone can aggravate DKK1 gene mRNA and protein levels inhibition (p *all* < 0.05), but exposure to As alone does not yield similar results (p *all* > 0.05). The expression of DKK1 gene mRNA and

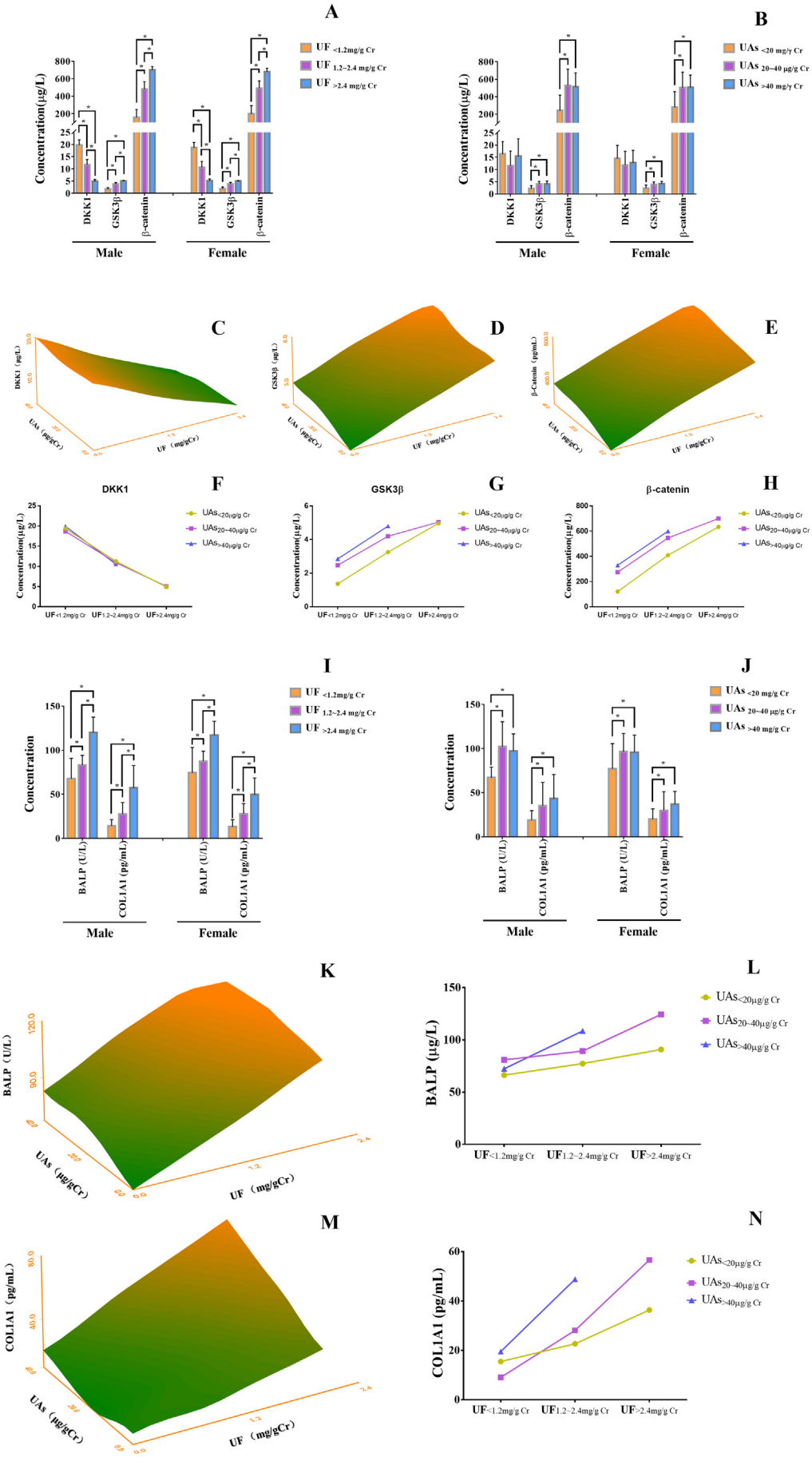

Fig. 1. Wnt signaling pathway is involved in osteogenic differentiation co-exposure to F and As in Chinese population. As the data shows, the results are consistent in male and female people that the differences of DKK1, GSK3β and β-catenin between the various fluoride exposed groups were significant. With the increase of F exposure level, the concentration of Wnt signaling inhibitor DKK1 gradually decreased, but the concentration of GSK3β and β-catenin increased (A). As can upregulate the GSK3β and β-catenin concentration, but the experiments did not detect any evidence that As can change the concentration of DKK1 in serum (B). As the concentration of bio-markers in the serum increases, the colour gradually changes from green to orange. As shown in panels C and F, F alone can affect the concentration of DKK1, but not As alone. F and As have no interaction with DKK1. Panels D and G show that both F and As can affect the concentration of GSK3β, and the difference in the interaction between F and As in GSK3β was statistically significant. Panels E and H show that F alone, As and their combination can affect the concentration of β-catenin. COL1A1 and BALP are indicators reflecting osteogenic differentiation in bone metabolism. As the exposure levels of F and As increased, the concentration of COL1A1 and BALP gradually increased, showing a significant dose-effect relationship (I, J). Both F and As can affect the content of COL1A1 and BALP, and an interaction exists between F and As on COL1A1 and BALP (K, L, M, N). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

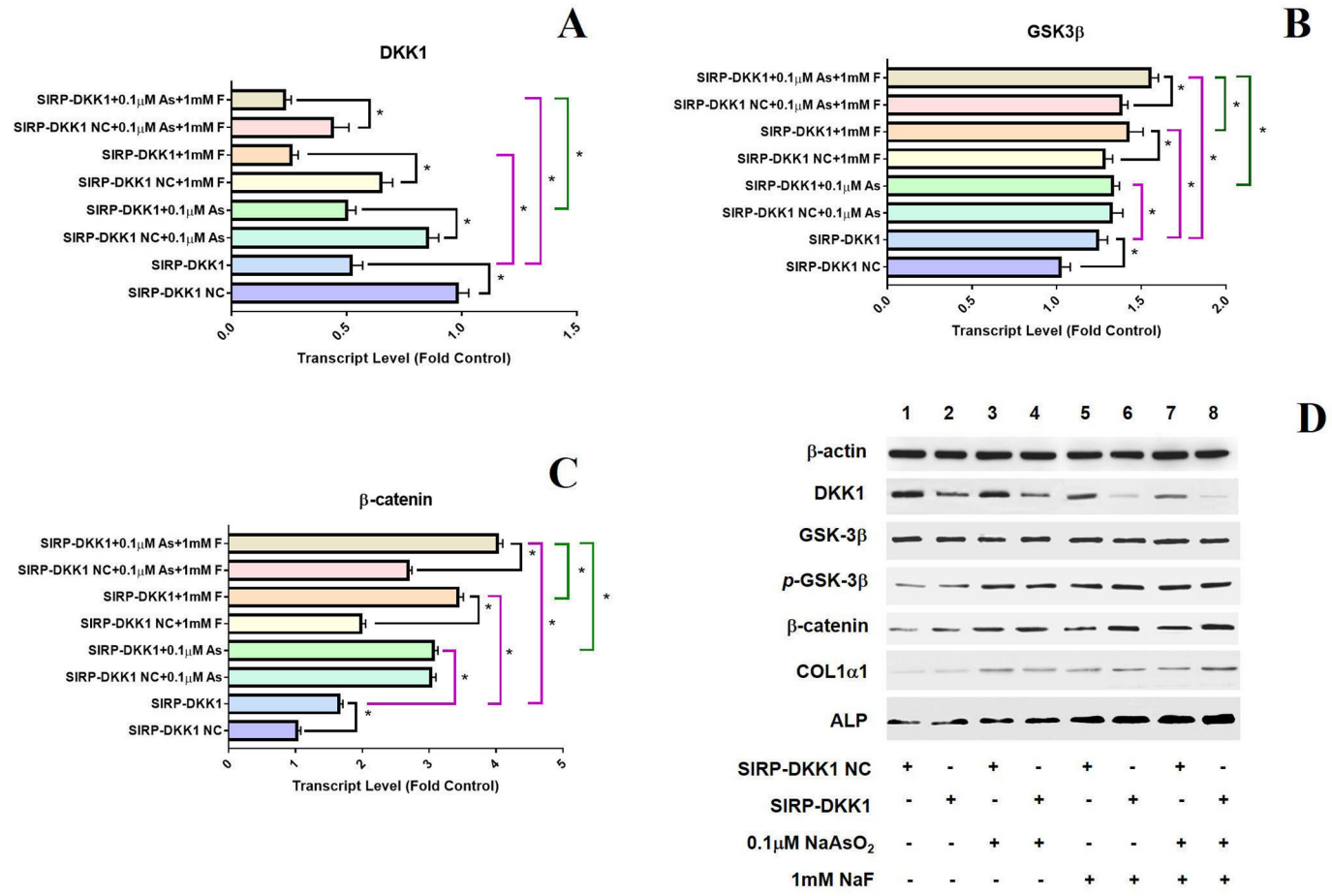

Fig. 2. Silencing DKK1 can promote osteogenic differentiation by increasing the activation of Wnt signaling caused by F, As and their combination. hFOB 1.19 cells was used to study the effects of F and As and their combination on the osteogenic differentiation. The DKK1 siRNA experiment shows that the expressions of DKK1 gene transcription expression (A) and protein level (D) were significantly decreased after silence the DKK1 gene. DKK1 transcription expression and protein level significantly decreased compared with SIRP-DKK1 NC group after F treatment, but not As (A, D). F and As alone and in combination can increase the transcription expression and protein levels of GSK3β (B, D) and β-catenin (C, D), and the protein levels of osteogenic differentiation indicators COL1A1 and BALP significantly increased (D).

protein levels in the group co-exposure to F and As was lower than that of the group single exposure to As ($p < 0.05$). Further analysis of other indicators of the Wnt signaling pathway, shown in Fig. 2B, C and D, show that F and As alone and in combination can increase the mRNA and protein levels of GSK3β and β-catenin, and the protein level of *p*-GSK3β protein is more obvious (*p all* < 0.05). The mRNA and protein levels of GSK3β and β-catenin in the combined exposure group were significantly higher than that in the exposed group of F and As alone (*p all* < 0.05). There are no significant differences in the mRNA and protein levels of GSK3β and β-catenin between the SIRP-DKK1 NC + 0.1 μM $NaAsO_2$ and SIRP-DKK1 + 0.1 μM $NaAsO_2$ groups (*p all* > 0.05). Fig. 3D shows the change in the protein level of the effective biomarker COL1A1 and BALP. F and As alone and in combination can increase the protein level of COL1A1 and BALP, after silencing DKK1 (*p all* < 0.05). The level of COL1A1 and BALP protein in the combined exposure group was significantly higher than that in the exposed group of F and As alone (*p all* < 0.05).

3.3. Silencing GSK3β can inhibit osteoblastic differentiation by decreasing the activation of Wnt signaling caused by F, As and their combination

As an important molecule of the canonical Wnt signaling pathway, the purpose of silencing GSK3β is to explore the role of GSK3β in osteoblastic differentiation caused by co-exposure to F and As. The results, as shown in Fig. 3B and D, the GSK3β transcriptional expression in the SIRP-GSK3β group was significantly lower than the SIRP-GSK3β NC group ($p < 0.05$). Compared with the SIRP-GSK3β NC group, the GSK3β protein expression level decreased by 40%, and *p*-GSK3β was reduced even more, reaching 70%.

After silencing GSK3β, co-exposure to F and As or single exposure to F can decrease the DKK1 gene mRNA and protein levels (*p all* < 0.05), but not As alone ($p > 0.05$). The expression of DKK1 mRNA and protein levels in the groups co-exposure to F and As or single exposure to F were lower than the group exposure to As alone (*p all* < 0.05). However, there was no difference in DKK1 mRNA and protein levels between the group co-exposure to F and As and the group exposure to F alone ($p > 0.05$). These results are shown in Fig. 3A and D. Fig. 3B and D show that only exposure to arsenic alone can increase the GSK3β mRNA and protein levels after silencing GSK3β (*p all* < 0.05), and the level of *p*-GSK3β protein is more obvious, but the GSK3β gene mRNA and protein levels (GSK3β and *p*-GSK3β) in the SIRP-GSK3β + 0.1 μM $NaAsO_2$ group were lower than those in the SIRP-GSK3β NC group (*p all* < 0.05). As shown in Fig. 3C and D, β-catenin gene mRNA and protein levels in F and As single or combined exposures were increased, but were lower than those of the SIRP-GSK3β NC group (*p all* < 0.05). Fig. 3D shows that the protein level of COL1A1 and BALP in F and As single exposure or combined exposure groups were slightly increased, but were lower than SIRP-GSK3β NC group.

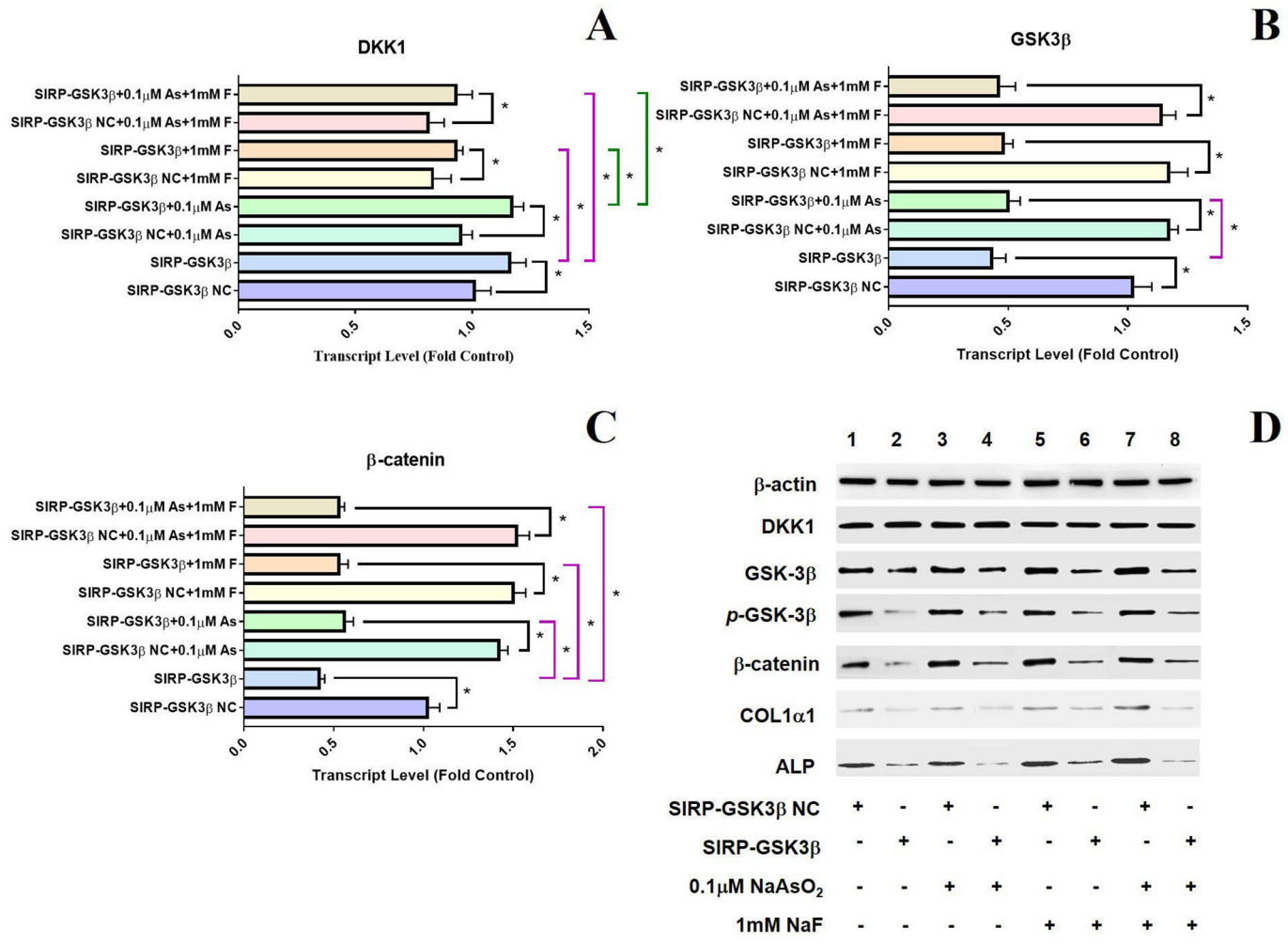

Fig. 3. Silencing GSK3β can inhibit osteogenic differentiation by decreasing the activation of Wnt signaling caused by F, As and their combination. The GSK3β siRNA experiment shows that the expressions of DKK1 gene transcription (A) and protein level (D) were significantly increased after silence the GSK3β gene, in contrast, the expressions of GSK3β gene transcription (B) and protein level (D) were significantly decreased. Another important finding was that exposure to F and As alone and combined could not reverse the inhibitory effect of GSK3β silencing on GSK3β (B, D) and β-catenin (C, D) expressions. And the protein levels of osteogenic differentiation indicators COL1A1 and BALP in F and As alone or combined exposure groups were lower than that of the SIRP-GSK3β NC group (D).

4. Discussion

Prior studies [21–25] that have noted the importance of the Wnt signaling pathway in the study of F and As alone; however, very little is known about the combined effect of these two chemicals, especially in the field of osteogenic differentiation. The first question in this study sought to determine the interaction of F and As on the Wnt signaling pathway and its role in osteogenic differentiation in the SEAF population. Three key proteins of Wnt signaling in serum exposed to different F and As were observed. As can be seen from the data, with the increase in exposure to F, the content of Wnt signaling inhibitor DKK1 gradually decreased, but the concentration of GSK3β, β-catenin and the important biochemical indicator COL1A1 and BALP for osteogenic differentiation were increased. This suggests that F can activate Wnt signaling by reducing the expression of the Wnt signaling inhibitor DKK1, thereby promoting bone formation. This is consistent with the results of previous studies [21,23,24]. Under control conditions, osteoblasts and osteoclasts maintain a certain amount and restrict each other, so that bone formation and bone resorption are in a state of dynamic equilibrium. The inhibition of DKK1 can break the above-mentioned equilibrium state, increase bone formation, and ultimately develop to bone sclerosis, which is one of the characteristic manifestations of skeletal fluorosis. Next, we grouped the SEAF population according to urinary As and found that As can upregulate the GSK3β, β-catenin concentration and the osteogenic differentiation bio-markers COL1A1 and BALP in serum. Another important finding was that the experiments did not detect any evidence that As can change the content of DKK1 in serum. Although the current data support the very little effect of As on DKK1, the downstream indicator of the Wnt signal has changed (including GSK3β and β-catenin). This suggests that As could induce the activation of Wnt signal by other pathways, which do not involve the inhibition of DKK1. Some studies [26–28] have shown that some members of the Wnt family can activate PKC through a non-canonical Wnt signaling pathway (Wnt/Ca^{2+}), thereby promoting phosphorylation inactivation of GSK3β. However, whether the non-canonical Wnt signaling pathway is involved in the regulation of As on classical Wnt signaling molecules remains to be further studied.

The interaction between chemicals is an important issue. To better understand the combined effects of F and As on the Wnt signaling pathway, we performed further interaction analysis. The results of our study show that F can decrease the concentration of DKK1 in serum; however, at the same exposure level of F, the DKK1 concentration did not change significantly as the exposure dose of As increased. This suggested that the interaction of F and As with DKK1 is mainly manifested by the independent effect of F. For GSK3β, the current results found that both F and As can affect the concentration of GSK3β in

serum. The concentration of GSK3β in $F_{low}As_{low}$ and $F_{low}As_{high}$ groups was higher than the group with single exposure to F (F_{low}) or As (As_{low} or As_{high} group), but less than the sum of the two effects. The concentration of GSK3β in $F_{high}As_{low}$ was higher than the As_{low} group, but there was no significant difference between the $F_{high}As_{low}$ and F_{high} groups. Simultaneously, the general linear regression model is demonstrated that the change of GSK3β is the result of the combined effect of the F and As. These results suggest that the interaction between F and As in the $F_{low}As_{low}$ and $F_{low}As_{high}$ groups can inhibit GSK3β. However, in the $F_{high}As_{low}$ group, the main effect is the independent effect of F. Finally, the results of this study show that the concentration of β-catenin COL1A1 and BALP in $F_{low}As_{low}$, $F_{low}As_{high}$ and $F_{high}As_{low}$ groups was higher than the group with exposure to F alone (F_{low} or F_{high} group) or As (As_{low} or As_{high} group), but less than the sum of the two effects. At the same time, the general linear regression model analysis demonstrated that there is an interaction with the concentration of β-catenin, COL1A1, BALP between F and As. These results suggest that the interaction between F and As can inhibit β-catenin and BALP in these exposure levels.

In short, the population-based study detected some evidence that the Wnt signaling pathway was involved in the bone damage caused by co-exposure to F and As. The interaction between F and As can inhibit the Wnt signaling pathway in these exposure levels, only DKK1 is manifested by the independent effect of F.

DKK1 is a target of Wnt signaling therapy, and anti-DKK1 (BHQ880 and DKN-01) monoclonal antibodies are being tested in clinical trials for cancer patients and osteoporotic post-menopausal women [29]. The population-based study showed that Wnt signaling pathway was involved in the osteogenic differentiation caused by co-exposure to F and As. But the key targets of the Wnt signaling pathway for osteogenic differentiation in combination with F and As is unknown. We attempted to silence the DKK1 gene, and then the Wnt signaling molecule mRNA and protein levels changes in hFOB 1.19 cells were observed to determine the role of DKK1 in the combined exposure of F and As. The DKK1 siRNA experiment showed that the expressions of DKK1 gene transcription and protein level were significantly decreased after silencing the DKK1 gene. DKK1 mRNA and protein levels significantly decreased compared with SIRP-DKK1 NC group after F treatment, but not As. And the expression level of DKK1 in the F-As combined exposure group was lower than that in the group exposure to As alone, but there was no significant difference in the group single exposure to F, it suggests that the interaction of F and As with DKK1 is mainly manifested by the independent effect of F. GSK3β and β-catenin mRNA and protein levels significantly increased after F and As alone and in combination treatment, it suggests that silencing DKK1 does not completely inhibit the stimulating effect of F and As alone and in combination on GSK3β and β-catenin. Moreover, F, As alone and in combination can up-regulate osteoblastic differentiation indicators COL1A1 and BALP protein levels, after silencing DKK1. These results demonstrated that silencing DKK1 can aggravate the inhibitory effect of F on DKK1, thereby increasing the expression of GSK3β and β-catenin and promoting osteoblastic differentiation. This finding is consistent with that of Zeng [30], who found that DKK1 can attenuate the anti-proliferative activity of F. Moreover, the results validate the hypothesis of population-based studies that arsenic has little effect on DKK1, and it is speculated that arsenic can promote osteoblastic differentiation through other pathways instead of DKK1-mediated classical Wnt signaling pathway.

GSK3β is a proline-directed serine-threonine kinase. In recent years, it has become an important therapeutic target for Wnt signaling [29]. To further study the role of GSK3β in F-As combined with bone damage, we attempted to silence the GSK3β gene, and then the Wnt signaling molecule mRNA and protein levels changes in hFOB 1.19 cells were observed. The GSK3β siRNA experiment showed that the expressions of GSK3β gene transcription and protein level were significantly decreased after silencing the GSK3β gene, but DKK1 mRNA and protein levels were increased. It is speculated that GSK3β inhibition can feedback regulate the expression of DKK1 and induce upregulation of DKK1. Another important finding was that exposure to F and As alone and combined could not reverse the inhibitory effect of GSK3β silencing on GSK3β and β-catenin. Moreover, the protein levels of osteoblastic differentiation indicators COL1A1, BALP in F and As single or combined exposure groups were lower than that of the SIRP-GSK3β NC group. These findings suggested that silencing GSK3β can inhibit osteoblastic differentiation by decreasing the activation of Wnt signaling caused by F, As and their combination.

5. Conclusions

In summary, we conclude that the Wnt signaling pathway is involved in the osteogenic differentiation caused by co-exposure to F and As, and the interaction between F and As can inhibit the Wnt signaling pathway in these exposure levels. Furthermore, silencing GSK3β instead of DKK1 can inhibit osteoblastic differentiation caused by co-exposure to fluoride and arsenic. Future studies will observe the role of overexpression of GSK3β and DKK1 in bone damage caused by F and As. In addition, as negative regulators of bone formation, it is necessary to explore the role of sclerostin (SOST) in osteoblastic differentiation induced by combined exposure of F and As. This study can provide a scientific basis for a further understanding of the causes of bone damage caused by F and As, and the improvement of targeted intervention strategies.

Conflict of interest statement

Nothing to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81472927), the Science and Technology Foundation of Guizhou Province (No. J [2015]2010).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.03.016.

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