Stromal-Cell-Derived Factor-1(SDF-1) Down-Regulates ANKH via NF-kB Promote Formation of Chondrocyte Mineralization.

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Abstract

Osteoarthritis (OA) is one of the most common forms of arthritis. However, the pathogenesis of OA remains unclear. Previous studies suggested that stromal-cell-derived factor (SDF)-1 was associated with some characteristic changes in cartilage. The aim of this study is, therefore, to explore the mechanism of SDF-1 promotes chondrocyte mineralization in OA. The effect of SDF-1 was analyzed with human C-28/I2 chondrocytes. The chondrocytes were transfected with pEX-4-ANKH or siRNA and treated with related inhibitor. The chondrocytes were subjected to Alizarin red S staining, PiPer phosphate assay kit, Alkaline phosphatase staining , RT-PCR and Western blot analysis. In vitro, SDF-1 markedly promoted the mineralization of chondrocytes and suppressed the expression of ANKH, the endogenous mineralization inhibitor. ANKH overexpressed in the chondrocytes significantly decreased the levels of the mineralization in response to SDF-1 treatment. Moreover, SDF-1 promoted an increase in the expression of p-TAK1 and p-IKKβ⍰p-IkB⍰p-NF-kB p65. Furthermore, SDF-1 induced decrease in ANKH expression was blocked by IKKβVI (IkB kinase inhibitor). To conclude, SDF-1 suppresses the expression of ANKH via activating NF-kB pathway to aggravate human chondrocytes calcification, suggesting blockade of SDF-1 might be a novel therapy for treatment of OA patients.

Introduction

Osteoarthritis(OA) is one of the most common forms of arthritis and is a chronic joint disease in the world, affecting about 18% of women and 10% of men over 60 years old(1). What make people debilitating are the loss of function and pain; the resulting socio-economic burden is very large, accounting for 1.0% to 2.5% of GDP in developed countries(2). Traditionally, in end-stage disease, the treatment of osteoarthritis includes relieve pain and joint replacement(3). This approach does not reduce the incidence of the early disease and the limitations of joint replacement surgery, including the limited lifespan of prostheses and the adverse postoperative complications. Prevention and treatment of early osteoarthritis require further understanding of the pathogenesis of the disease.

The pathogenesis of the disease is complex and composed of many factors, including genetics, biology and biomechanics. Osteoarthritis was once considered as a purely mechanical cartilage degeneration disease, but now it is known that it is a complex condition affecting the whole joint, in which the activation of MMP plays a key role(4). Synovium, cartilage and subchondral bone may play a key role in the pathogenesis of the disease. More and more evidence shows that cartilage destruction and cartilage mineralization, subchondral osteosclerosis, synovitis and osteophyte formation are more important than previously thought(5). Although the etiology and pathology of OA are still elusive, it is considered to be multifaceted , mainly including abnormal mineral reactions of articular cartilage, resulting in biomechanical overload or tendency of triggering age, manifested as excessive basic calcium phosphate (BCP) crystals in late-stage OA(6). Furthermore, the response of cartilage mineralization occurs as part of human OA but is not a variable feature of the disease(7). As an important mediator regulating mineral response (BCP crystals), multipass transmembrane ankylosis transporter (ANK), transports intracellular PPi to the extracellular environment, participates in the pathogenesis of chondrocyte mineralization,

which is mainly due to the imbalance of effector and regulatory factors(8). However, the regulatory mechanism of ANKH in the mineralization of articular chondrocytes is not fully understood.

In recent years, Stromal-cell-derived factor-1(SDF-1) has been paid more and more attention in the pathogenesis of OA(9). SDF-1 is associated with some characteristic changes in bone and cartilage (10). The level of SDF-1 in OA synovial fluid increased, and the concentration of SDF-1 in serum decreased significantly after synovectomy(11). Data reported by some research groups suggest that the SDF-1 may be a catabolic regulator of cartilage destruction in osteoarthritis. However, SDF-1 appears to regulate the chondrogenic differentiation of bone marrow stromal cells(BMSCs) induced by BMP-2 and enhances the proliferation and maturation of chondrocytes(12). In addition, it can also induce BMSCs to migrate to the joint lesions(13). Therefore, the effects of SDF-1 on cartilage remain inconclusive.

Given the important role of SDF-1 in OA, this study was undertaken to determine if SDF-1 stimulates the expression of ANKH and involving its signaling pathway in articular chondrocytes of mineralizing.

Materials And Methods

Cell culture

Articular cartilage and costal cartilage are both of permanent hyaline cartilage tissues, which have to supply cartilage matrix components and should never go beyond prehypertrophic stages in chondrocytic differentiation, except under pathological conditions. C-28/I2, the immortalized chondrocyte cell lines, transduced with simian virus 40 (SV 40) containing the large T-antigen were originated from rib cartilage of a 15-year-old female(14). Cells were seeded at a density of 100,000 cells/cm^2 in 25 cm^2 flasks and cultured in DMEM/F-12 medium (HyClone SH30023.01) containing 10% fetal calf serum (PAN Biotech P30-3302) at 37°C and 5% CO_2.

Overexpression and siRNA transfection of ANKH

Briefly, the chondrocytes were transfected with pEX-4-ANKH or siRNA (GenePharma) which were added to the total amounts of transfected DNA samples using Lipofectamine2000 according to the manufacturer's protocol. Controls use nontransfected cells in the same way of transfection. Cells were cultivated for 24h and then collected for further experiments.

Pi uptake by chondrocytes.

PiPer phosphate assay kit (Molecular Probes, Eugene, Oreg) measured the intracellular Pi concentration of chondrocytes. Cells were washed, and the cytoplasmic fraction was obtained by ultracentrifugation as described below[15]. According to the manufacturer's instructions, the Pi concentration was measured by ten microliters of the cytoplasmic fraction.

Alkaline phosphatase staining

The chondrocytes were inoculated into six-well plates at a density of 1×10^5 cells per well. After the cells reached 70% confluence, the alkaline phosphatase (ALP) secretion was measured by the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) staining the samples according to the manufacturer's instructions.

Alizarin red S staining

For estimation of mineralization event, confluent cultures of chondrocytes were preincubated in DMEM/F-12 containing 0.5% FBS for 24 h and then incubated in the same medium with SDF-1α(PeproTech), AMD3100(Sigma) or IKKβVI(Sigma). PBS was added to the control culture. Cells were stained with ALIZARIN RED S assay kit (GENMED) according to the manufacturer's instructions. To solubilize and release calcium-bound alizarin red into solution, the Alizarin red S stained cultures were incubated with 100 mM cetylpyridinium chloride for 2 h. Spectrophotometer was used to measured the absorbance of the released alizarin red S at 570 nm. Data are expressed as units of alizarin red S released per milligram of protein in each culture.

Real-time polymerase chain reactions (RT-PCR)

Total RNA was extracted by the TRIzol (Solarbio) method. According to the manufacturer's instructions (TaKaRa), RNA was reverse-transcribed to cDNA. The TB Green Premix Ex Taq™ II (TaKaRa) was used to measured the expression levels of ANKH, col X, col□ and β-actin at the mRNA level. The following cycling conditions of RT-PCR were utilized: 95℃ for 30s and 40 cycles at 95℃ for 10s and at 60℃ for 30s. The primers for RT-PCR are listed in Table1. The expression of mRNA was calculated by 2-Δ Δct method, and ACTB was used as a reference gene.

Western blot analysis

RIPA lysis buffer (Solarbio) was used to extract cellular protein, and protein concentration was measured with the BCA Protein Assay kit (Solarbio). The extracted cellular protein was loaded on SDS-PAGE to electrophoresis, then it was transferred to a nitrocellulose membrane and incubated with 4% BSA in TBST for 60 min to block non-specific binding. The membrane was then incubated with the primary antibodies overnight at 4℃ in 4% BSA/TBST. The primary antibodies included anti-TAK1(1:1000, 4505, Cell Signaling Technology), anti-p-TAK1(1:1000, 4537, Cell Signaling Technology), anti-IKKβ(1:1000, 2684, Cell Signaling Technology), anti-p-IKKβ(1:1000, 2694, Cell Signaling Technology), anti-IkBα(1:1000, 9242, Cell Signaling Technology), anti-p-IkBα(1:1000, 9247, Cell Signaling Technology), anti-p-NF-kB p65(1:1000, 3031, Cell Signaling Technology), anti-ANKH(1:1000, ab129001, Abcam) and anti-GAPDH(1:1000, ab8245, Abcam). The membranes were incubated with anti-rabbit or anti-mouse secondary antibody for 60 min at room temperature. Finally, we measured the proteins on the membranes by a chemiluminiscent signal generated using Western blot detection reagents (Fdbio science). Western blot was visualized by enhanced chemiluminescence with Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Statistical analysis

One-way analysis of variance (ANOVA) was used for multifactorial comparisons in this study. Experiments were performed three times. Data are presented as mean ± SD. Values of P < 0.05 were considered statistically significant. All data analysis was conducted with SPSS 22.0 analysis software.

Results

SDF-1 regulates the mineralization of chondrocytes.

Since SDF-1 was reported to be upregulated in patients with OA, we then sought to evaluate whether SDF-1 could regulate the role of mineralization events in chondrocytes. Given that levels of intracellular Pi uptake have been used to measure mineralization events in chondrocytes(16), we assessed Pi uptake in cells stimulated with SDF-1. We found that levels of Pi were increased with the increase of SDF-1 concentration and significantly enhanced at the concentration of 80 ng/ml and peaked at the concentration of 100 ng/ml, indicating that SDF-1 regulates levels of intracellular Pi uptake in cells in a dose-dependent manner (Fig. 1A). Moreover, we also observed that SDF-1 induced levels of Pi uptake at the peak concentration after 24 h, indicating that SDF-1 regulates levels of intracellular Pi uptake in a time-dependent manner (Fig. 1B). Furthermore, alizarin red S staining and quantitative alizarin red binding assay were consistent with the result of intracellular Pi uptake. It further revealed that the stimulatory effect of SDF-1 on chondrocytic matrix mineralization is a time-dependent manner (Fig. 1C and D). In addition, given that several biomarkers markers have been found to be correlated with cartilage mineralization(17), we next performed real-time PCR to investigated whether SDF-1 could regulate differentiation events in chondrocytes. Cells treated with SDF-1 showed a significant increase of col∥ a1 mRNA levels in a dose-dependent manner. However, we found that there was no statistical difference in col∥ a1 mRNA levels between control and SDF-1 group(Fig. 1E). ALP expression was reported to be upregulated in matrix mineralization of chondrocytes(18), we analyzed whether SDF-1 could also regulate the expression of ALP. We found that SDF-1 could regulate the ALP expression(Fig. 1F).Taken together, these results suggest that stimulatory effect of SDF-1 is involved in the regulation of chondrocyte mineralization.

SDF-1 regulates the mineralization of chondrocyte through CXCR4

The CXCR4 is a receptor for SDF-1 in primary chondrocytes. Cells cultured with different concentrations of AMD3100, a SDF-1/CXCR4 axis inhibitor, show a significant decrease of chondrocytic matrix minerals in response to SDF-1 treatment in a dose-dependent manner(Fig. 2A). Pyrophosphate (an inhibitor of calcification) efflux to the extracellular matrix was suppressed along with the decreased expression of ANKH, a transmembrane protein that controls pyrophosphate efflux of cells. We then sought to evaluate whether SDF-1 could regulate the expression of ANKH in chondrocyte through CXCR4. To this end, cells were cultured with SDF-1 in the presence and absence of AMD3100, respectively. The results showed that ANKH protein and mRNA expression markedly decreased in SDF-1 treatment group compared with the control group and AMD3100 blocked SDF-1-induced decrease in ANKH expression(Fig. 2B and C). These

data indicate that the mineralization of chondrocyte was achieved by SDF-1 interaction with its receptor CXCR4 in chondrocytes.

SDF-1 regulates the mineralization of chondrocytes though ANKH expression

After confirming that SDF-1 downregulates the expression of ANKH in chondrocytes, we further investigate the mechanisms involved in SDF-1 induced mineralization events. To this end,

the ANKH gene and GFP or only GFP were transfected in the chondrocytes with lentiviral vectors, and stable transgenic Control and ANKH cells were sorted and confirmed by immunofluorescence(Fig. 3A). The expression of ANKH was measured at both the protein and mRNA level(Fig. 3B and D), the results of which suggested that the ANKH gene was successfully transfected in cells. Moreover, ANKH-knockdown cells were constructed with a ANKH-specifc siRNAs, and the protein (Fig. 3C) and mRNA (Fig. 3E) expressions of ANKH significantly decreased than negative-siRNA group or control group cells. Then, we assessed the level of Pi uptake and mineralization in chondrocytes. The results showed that the levels of Pi uptake in siRNA group were significantly higher than those in ANKH overexpressed treatment group. We found that significant decreases in the levels of Pi uptake in ANKH overexpressed treatment group compared with control group in response to SDF-1 treatment(Fig. 3F). In addition, this observation was further supported by alizarin red S staining and quantitative alizarin red binding assay results(Fig. 3G). These results show that SDF-1 regulates the mineralization of chondrocytes though ANKH expression.

SDF-1 regulates the expression of ANKH via activating NF-kB pathway

We further investigate the mechanisms involved inSDF-1 induced ANKH expression. Since NF-kB signaling was reported to aggravate human vascular calcification via repression of ANKH expression(19). we then sought to evaluate whether SDF-1 could regulates the expression of ANKH via activating NF-kB pathway. To this end, C-28/I2 cells were cultured with SDF-1 in the absence or presence of AMD3100. We found that significant increases in the levels of p-TAK1 and p-IKKβp-IkBp-NF-kB p65 in the SDF-1 treatment group compared with the control group, which were further decreased by AMD3100 group (Fig. 4A). To further test whether SDF-1 could regulate the expression of ANKH in chondrocyte via activating NF-kB pathway. Cells were cultured with SDF-1 in the presence and absence of IKKβVI (IkB kinase inhibitor). In accordance, SDF-1 induced decrease in ANKH expression was blocked by IKK inhibition (Fig. 4B). And this observation was further supported by alizarin red S staining and quantitative alizarin red binding assay results(Fig. 4C). Therefore, SDF-1 may regulate the expression of ANKH via activating NF-kB pathway to promote calcification.

Discussion

The key risk factors may vary by joint and disease stage. It is difficult to distinguish disease development or progression from related single and clustered risk factors(20). Osteoarthritis is a disease caused by multiple factors such as gender, age, genetic factors, biomechanical changes, BMI, nutritional factors, and bone density(21).

Previous studies have shown that SDF-1 plays vital roles in cell migration, HIV-1 infection and embryonic development(22). We noticed that in cartilage degeneration SDF-1 has been reported to play an important role by infiltrating chondrocytes(23), and that synovectomy could improve the onset of OA(24). In this study, we found that the SDF-1 may play a role in the chondrocyte mineralization events of OA. Our study provides a novel mechanism of SDF-1 exacerbating human cartilage mineralization by activating NF-kB. The endogenous inhibitor of mineralization ANKH expression was suppressed by SDF-1-activated NF-kB in vitro. We provide a direct link between SDF-1 and the osteogenic process attributed to cartilage mineralization.

Our data demonstrates that SDF-1 not only stimulates mineralization of chondrocytes but also hypertrophic differentiation of chondrocytes. Our results show that treatment of SDF-1 upregulated hypertrophic differentiation markers, including type X collagen in the chondrocytes, but there was no statistical difference in proliferation and maturation markers, including type Ⅱ collagen. We believe that these results are accurate because they are close to these previous studies(25). Studies have also confirmed that, in addition to SDF-1, other chemokines such as CXCL8 and CXCL1 promote chondrocytes mineralization(26, 27).

Further studies indicate that the effect of mineralization could be explained by NF-kB activation at 100 ng/mL SDF-1. Functional studies combined with Western blots show upstream pathway involving the activation of TAK1. TAK1 is the key upstream regulator of the NF-kB pathway(28). This activation seems to be followed by the phosphorylation of IKKβ. Our observations show that TAK1 phosphorylates IKKβ, in accordance with published studies(29). The IKKβ then not only targets the IκB inhibitor protein but also phosphorylates the p65 NF-κB subunit, in agreement with previous observations(30). Importantly, AMD3100, CXCR4 antagonist, was observed to markedly suppress the upregulation of TAK1 and p65 expression induced by SDF-1, indicating that SDF-1 activates NF-kB pathway through CXCR4.

Owing to its ability to regulate functions of chondrocytes and osteoblasts, ANKH is involved in the pathogenesis of osteoarthritis(31). In the current study, we found that overexpressed ANKH could inhibit the mineralization of chondrocytes stimulated by SDF-1. And suppression of ANKH expression in chondrocytes with siRNA was sufficient to accelerate the mineralization of chondrocytes stimulated by SDF-1. In addition, we found that SDF-1 markedly increased ALP activity, this result matched others(32), which was a necessary role to permit vertebrate biomineralization. These findings suggest that SDF-1 could regulate the mineralization of chondrocytes though ANKH expression.

NF-kB activation has been shown to up-regulate many prometastasis genes(33). Similar researches have shown that NF-kB activation by SDF-1 leads to up-regulation of CXCR4, thereby increasing cell proliferation and differentiation(34). Recent study has shown that NF-kB accelerates vascular calcification by inhibiting the expression of ANKH(19). ANKH, a multi-channel transmembrane protein, could export inorganic PPi to the extracellular space, inhibit the formation of hydroxyapatite, and prevent calcium deposition in skeletal tissue(35). Mutations in ANKH cause two different calcification disease: craniometaphyseal dysplasia(36) and familial calcium pyrophosphate dihydrate deposition disease(37).

Some research suggests that ANKH mRNA expression is lower in the hypertrophic chondrocytes of the murine growth plate, and ANKH expression is downregulated in the cartilage of osteoarthritis, where mineralization also occurs(38, 39). In our study of chondrocytes, SDF-1 could downregulate ANKH expression, and this down-regulation is inhibited by the AMD3100. Moreover, we found that ANKH expression in chondrocytes was significantly increased after IKKβVI, IkB kinase inhibitor, treated. These data indicate that SDF-1 markedly repressed ANKH expression via activating NF-kB pathway.

In conclusion, we provide a direct evidence that SDF-1 suppresses the expression of ANKH, the endogenous mineralization inhibitor, via activating NF-kB pathway to aggravate human chondrocytes calcification. Our data may shed light on understanding the complexity of chondrocytes calcification during OA. It is widely known that bisphosphonates can inhibit mineralization formation. Bisphosphonate therapy has been identified as an effective treatment for the patients or different animal models of OA, but there is a great portion of them unresponsive to bisphosphonate whereby the mechanisms are not completed investigated(40). Therefore, it is necessary to find out a new approach to the treatment of Bisphosphonate-unresponsive OA patients. In our study, we found that inhibition of SDF-1 could also reduce the levels of mineralization and increase ANKH expression in chondrocytes in vitro, indicating that blockade of SDF-1 might be a novel therapy for treatment of OA patients who fail to respond to Bisphosphonate.

Declarations

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors report no conflict of interest.

Acknowledgments

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Tables

Table 1. The primers sequences used for qRT-PCR

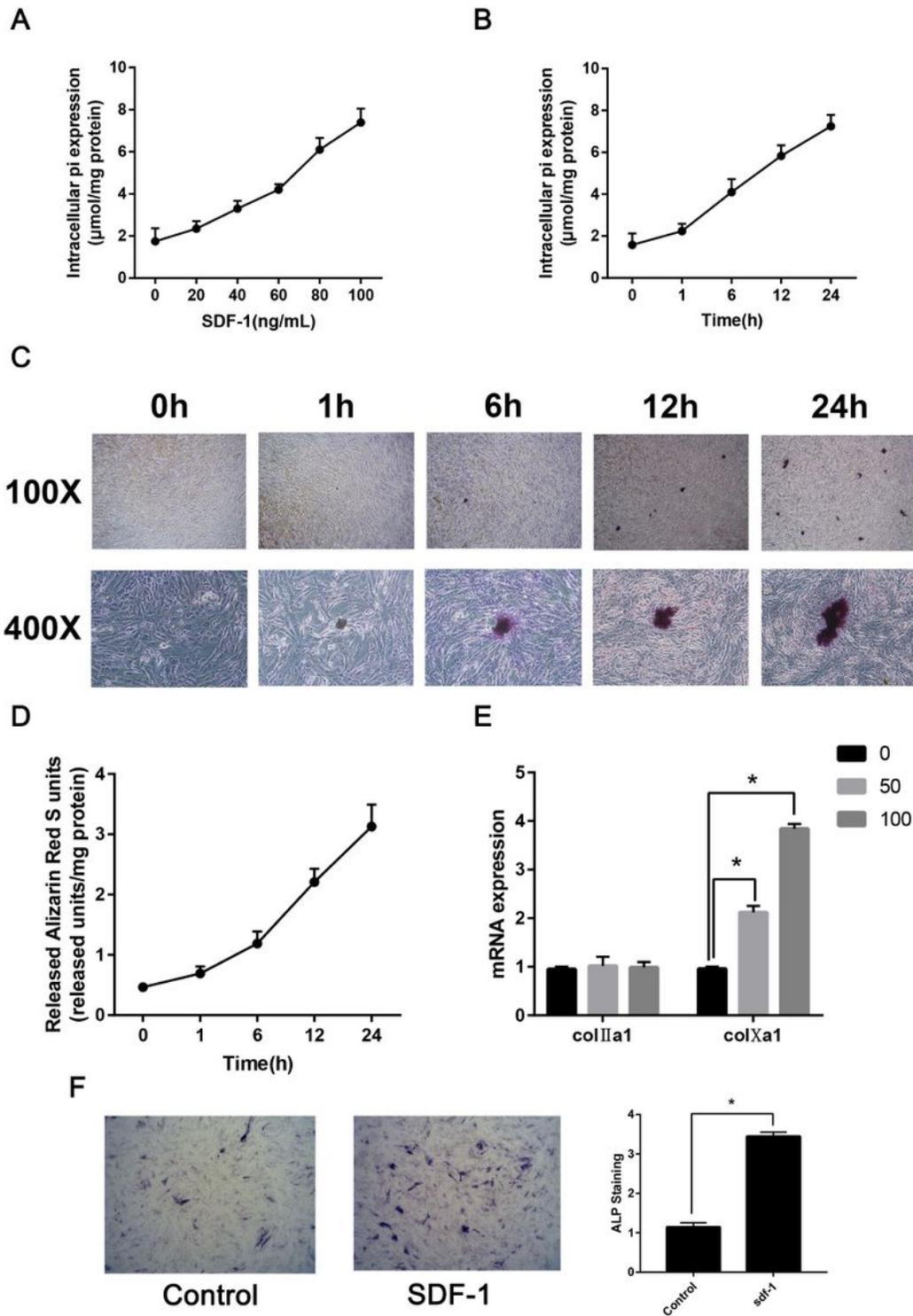| Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ANKH	ACAGAGGCAGTGGCGATTTT	GTTTGTTGCTGGGGTTATTCTTG
Col□	AGACTGGCGAGACTTGCGTCTA	ATCTGGACGTTGGCAGTGTTG
Col X	GACTCATGTTTGGGTAGGCCTGTA	CCCTGAAGCCTGATCCAGGTA
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

Figures

Figure 1

SDF-1 regulates the mineralization of chondrocytes. (A, B) Levels of Pi uptake in chondrocytes was treated with SDF-1 (0, 20, 40, 60, 80 and 100 ng/ml) and with SDF-1 (100 ng/ml) in different time period (0,1, 6, 12 and 24 h). (C, D) Alizarin red S staining of extracellular matrix mineralization in chondrocytes was treated with SDF-1 (100 ng/ml) in different time period (0, 1, 6, 12 and 24 h), original magnification ×100 and ×400. (E) RT-PCR analysis of colⅡa1 and colⅩa1mRNA expression in chondrocytes was treated

with SDF-1 (0, 50 and 100 ng/ml). (F) ALP stain analysis of ALP expression. Data are expressed as mean ± SD (n=3). *P < 0.05. Scale bars, 50 μm.
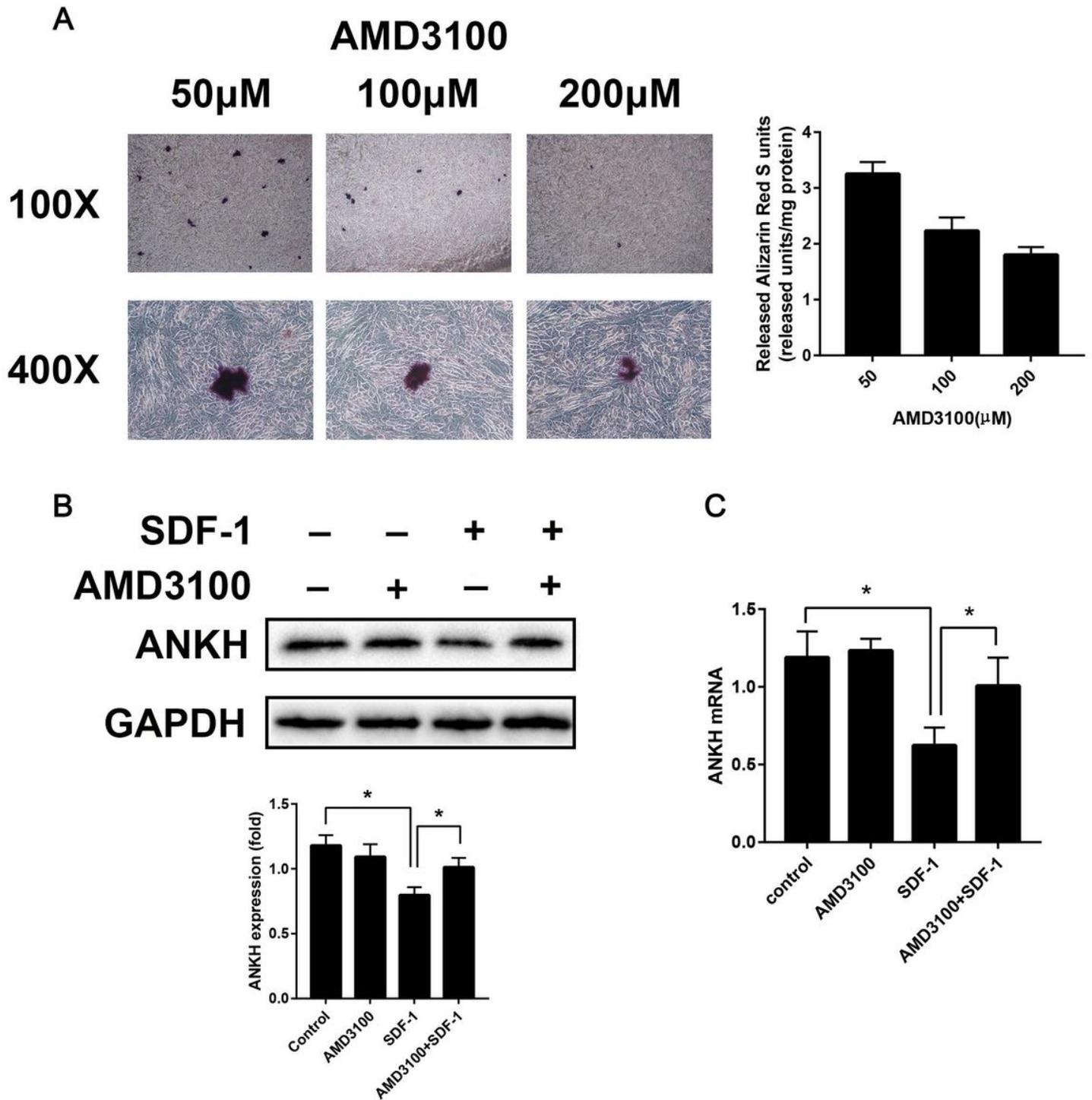

Figure 2

SDF-1 regulates the mineralization of chondrocyte through CXCR4. The chondrocytes were incubated with different concentrations of AMD3100 (50μM, 100μM, 200μM) for 2 h, and then cultured with SDF-1(100 ng/ml) for 24 h, (A) Alizarin red S staining assay of extracellular matrix mineralization. The chondrocytes were incubated with or without AMD3100(100μM) for 2 h, and then cultured with or without

SDF-1(100 ng/ml) for 24 h, (B) Western blot analysis of ANKH expression, (C) RT-PCR analysis of ANKH mRNA expression. Original magnification ×100 and ×400. Data are expressed as mean ± SD (n=3). *P<0.05. Scale bars, 50 μm.
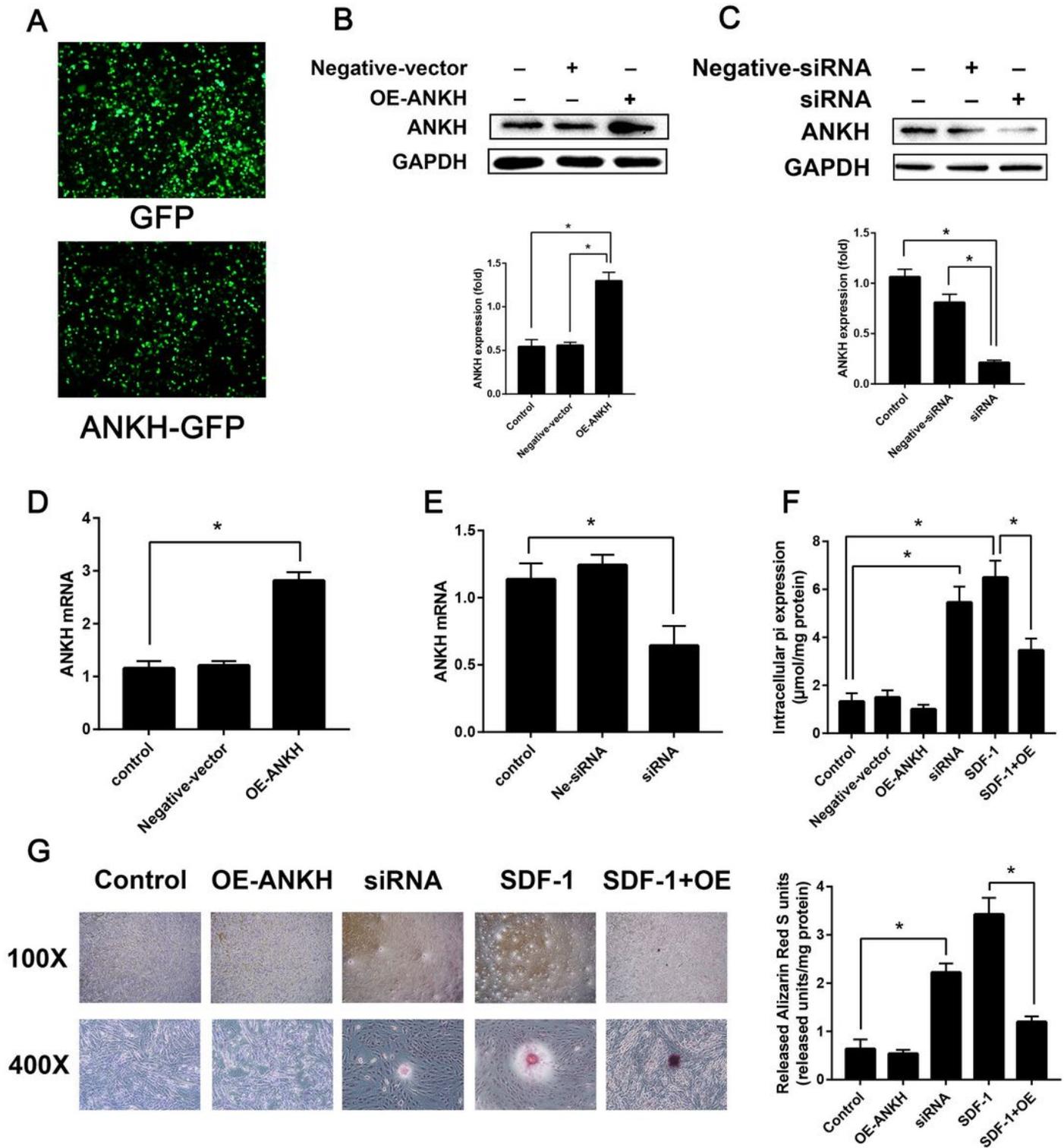

Figure 3

SDF-1 regulates the mineralization of chondrocytes though ANKH expression. (A)The cells were successfully transfected with lentiviral vectors, as indicated by immunofluorescence. The expression of

ANKH in transgenic chondrocytes was measured at (B, C) protein and (D, E) mRNA level. (G) Alizarin red S staining assay of extracellular matrix mineralization, and (F) levels of Pi uptake. Original magnification ×100 and ×400. Data are expressed as mean ± SD (n=3). *P<0.05. Scale bars, 50 μm.
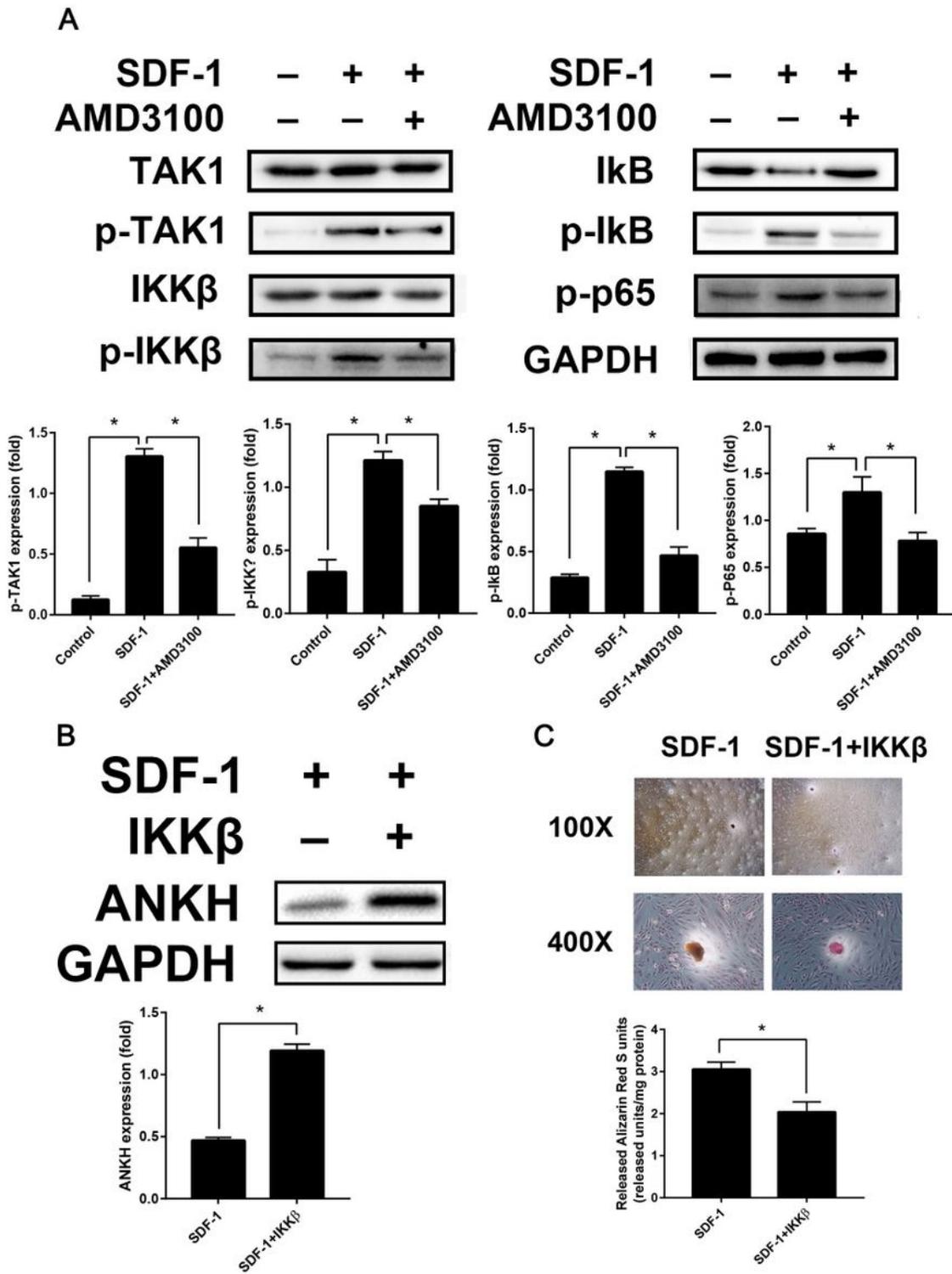

Figure 4

SDF-1 regulates the expression of ANKH via activating NF-kB pathway. (A)The cells were incubated with or without AMD3100 (100 μM) for 1 h, and then cultured with SDF-1 (100 ng/ml) for 24 h, Western blot

analysis of TAK1, p-TAK1, IKKβ, p-IKKβ, IkB, p-IkB and p-P65 expression. (B)The cells were cultured with SDF-1(100 ng/ml) in the absence or presence of IKKβVI(40 nM), Western blot analysis of ANKH expression. (C)Alizarin red S staining assay of extracellular matrix mineralization. Original magnification ×100 and ×400. Data are expressed as mean ± SD (n=3). *P<0.05. Scale bars, 50 μm.
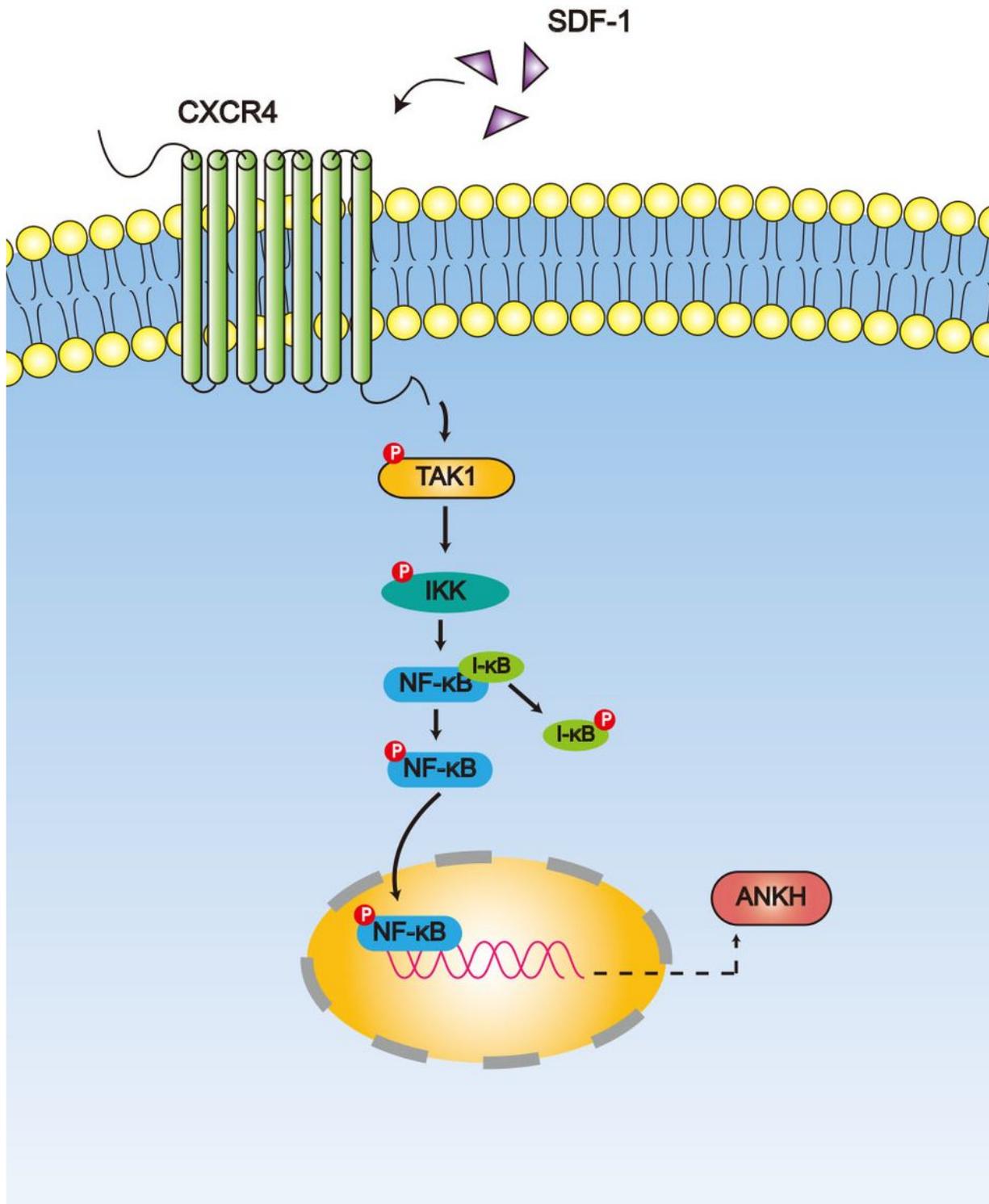

Figure 5

Schematic representation of the regulation of ANKH expression by SDF-1 in the chondrocytes. Shown is the involvement of TAK1 and then to phosphorylated IKK following the activation of NF-kB. Activation of NF-kB as translocation into the nucleus and subsequent repression of the ANKH expression.