



Strontium Regulates the Proliferation and Differentiation of Isolated Primary Bovine Chondrocytes *via* the TGF β /SMAD Pathway

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The present study evaluated the effects of strontium (Sr) on proliferation and differentiation of chondrocytes isolated from dairy cows, and whether Sr exerts its effects *via* transforming growth factor β (TGF β) signaling. The chondrocytes were isolated from patellar cartilage from newborn Holstein bull calves ($n = 3$, 1 day old, 38.0 ± 2.8 kg, fasting) within 15 min after euthanasia, and treated with different concentrations of Sr (0, 0.1, 1, and 10 $\mu\text{g/ml}$, as SrCl₂·6H₂O). After pretreatment with or without activin receptor-like kinase 5 (ALK5) inhibitor (10 μM SB-505124) for 4 h, chondrocytes were incubated with Sr for another 4 h. Overall effects of Sr were evaluated relative to NaCl as the control. In contrast, the 1 $\mu\text{g/ml}$ Sr-treated group served as the control to determine effects of preincubating with SB-505124. Western blot and qRT-PCR were used for measuring expression of proliferation-, differentiation-, and TGF β 1-responsive factors. Data were analyzed using one-way ANOVA in GraphPad Prism 7.0. Incubation with all doses of Sr increased TGF β 1/ALK5-induced SMAD3 phosphorylation, and at 10 $\mu\text{g/ml}$ it inhibited ALK1-induced SMAD1/5/9 phosphorylation. Expression of mRNA and protein of the proliferation-responsive factors type II Collagen α 1 (COL2A1) and aggrecan (ACAN) was induced by Sr at 1 $\mu\text{g/ml}$. In contrast, Sr at 10 $\mu\text{g/ml}$ inhibited the expression of differentiation-responsive factors type X Collagen α 1 (COL10A1) and secreted phosphoprotein 1 (SPP1), and at 1 $\mu\text{g/ml}$ it had the same effect on alkaline phosphatase (ALPL) mRNA and protein levels. Cells were stained with PI/RNase Staining buffer to assess cell cycle activity using flow-cytometry. Incubation with Sr at 1 and 10 $\mu\text{g/ml}$ induced an increase in the number of cells in the S-phase, leading to an increase in the proliferation index. Incubation with SB-505124 inhibited phosphorylation of SMAD3. Abundance of ACAN and COL2A1 mRNA and protein was lower when cells were pre-incubated with SB-505124. Overall, data indicated that Sr promotes proliferation and inhibits differentiation of primary chondrocytes by directing TGF β 1 signaling towards SMAD3 phosphorylation rather than SMAD1/5/9 phosphorylation. Whether these effects occur *in vivo* remains to be determined and could impact future application of Sr as an experimental tool in livestock.

Keywords: strontium, bovine chondrocyte, proliferation and differentiation, TGF β , Smad3

INTRODUCTION

Strontium (Sr) belongs to the second major group of elements, along with Ca and Mg (Pilmane et al., 2017). Although present in trace amounts in the body, similar to Ca, Sr is also a bone-seeking element due to its similar physicochemical properties. Most of the Sr entering the body is absorbed by bones and teeth (Nielsen, 2004). The function of Sr in the context of bone relates to its role in promoting osteoblast-mediated bone formation and inhibiting osteoclast-mediated bone resorption (Querido et al., 2016).

In ruminants, it is well-established that Ca absorption from the gastrointestinal tract, Ca reabsorption from the kidney and mobilization of skeletal Ca stores help maintain Ca concentrations in the blood (Hernandez-Castellano et al., 2020). Thus, because Ca levels in the blood do not reflect intestinal absorption capacity, protocols for using Sr as a surrogate marker have been developed (Milsom et al., 1987; Khan et al., 2013). Studies with cows and sheep have reported a close correlation between the absorption rates of oral Sr and radioactive Ca, indicating that the Sr concentrations in the blood measured orally can serve as an index for Ca absorption capacity of the gastrointestinal tract of dairy cows and sheep (Hyde and Fraser, 2014; Hyde et al., 2019). Although these studies have provided information on the use of Sr, it is unknown to what extent (if any) Sr can affect other tissues in the body.

The transforming growth factor β (TGF β) superfamily comprises more than forty members including TGF β , activin, and bone morphogenetic protein (BMP) (Chen et al., 2012) all of which play pivotal roles in the metabolism, differentiation, proliferation, and survival of chondrocytes (van Caam et al., 2016; Woods et al., 2021). SMAD family member (SMAD)-dependent signaling is a classical pathway of the TGF β family and it involves binding of TGF β to its tetrameric receptor comprised of activin receptor-like kinase 5 (ALK5) and TGF β type II kinase receptor dimers. As a result, the signal delivered into cells induces subsequent phosphorylation of the receptor-SMAD components, SMAD2 and SMAD3 (Zhu et al., 2016; Thielen et al., 2019). These receptor-SMAD complex binds to the common-SMAD, SMAD4, to trigger nuclear translocation of this whole complex to regulate gene transcription. Together with the

phosphorylation of SMAD1/5/9 *via* ALK1, these events (at least in non-ruminants) regulate cartilage and bone development as well as homeostasis (Wu et al., 2016).

A previous study reported that Sr can regulate proliferation and differentiation of chondrocytes in rats by promoting the expression of TGF β 1 and TGF β 2 (Kong et al., 2018). However, it is unknown if Sr can mediate the TGF β pathway to regulate chondrocyte proliferation and differentiation in the bovine. Thus, the main objective of the present study was to determine *in vitro* the effect of Sr on proliferation and differentiation of bovine chondrocytes *via* the TGF β signaling pathway.

MATERIALS AND METHODS

Ethics Statement

This study was conducted at one of the experimental farms of Northwest A&F University (Shaanxi Province, China) in Western China (106°55'57"E, 34°48'41"N). The protocol was approved by the Animal Welfare and Research Ethics Committee at Northwest A&F University (Permit Number: 2021049), Shaanxi, People's Republic of China.

Animals and Tissue Collection

Tissue was isolated from newborn Holstein bull calves ($n = 3$; 38.0 ± 2.8 kg BW). At each of 3 consecutive d, within 15 min after euthanasia by a veterinarian with barbiturate, patellar cartilage was separated from the articular knee of each calf through surgical patellar excision. Patellar cartilage was then washed three times with 0.1% PBS and within 1 h transported to the laboratory on ice.

Isolation and Culture of Bovine Primary Chondrocytes

Primary chondrocytes were isolated from patellar cartilage from each calf and cultured individually. Methods for isolation of chondrocytes were described in a previous study (Wang et al., 2013). Briefly, the cartilage was sliced into thin slices and

TABLE 1 | Sequences of the primers used in this study.

Gene ^a	NCBI reference sequence	Product length (bp)	Forward primer	Reverse primer
ALK5	NM_001130916	323	TTCGGTGAGGCGAGAGATT	GCAATACAGCAAGTTCACCT
TGF β 1	NM_001166068	159	GACACCAACTACTGCTTCA	ATCCAGGCTCCAGATGTAA
SERPINE1	NM_174137.2	381	GGCTCAGACCAACAAGTT	TTCACCTCAATCTTCACCTT
ID1	NM_001097568.2	73	GCTCCGCTCAGCACTCTCAA	GATCGTCCGCTGGAACACA
ALK1	NM_001083479.1	109	ACAACACAGTGCTGCTCAGACA	TGCTCGTGGTAGTGCGTGAT
COL2A1	NM_001001135.3	299	GTGGAAGAGCGGAGACTA	GGTAGGTGATGTTCTGAGAG
ACAN	NM_173981	155	CGGAAGTGAGTGAGAGT	GGTGGTCTGATGACAATA
ALPL	NM_176858.2	163	AACACAAGCACTCTCACTAT	GCCATCTCTACCATCTCAG
COL10A1	NM_174634.1	184	AGCTGAGATCATGCTGCCAC	CTCTCCTCTCAGTGATACACCTTT
SPP1	NM_174187	158	AGAGGAGGACTTCACATCA	TCAGATTGGAATGCTTGTTC
VEGFA	NM_001316955	270	CCTTGCTGCTTACCTTC	TGGTGATGTTGAACTCCTC
GAPDH	NM_001034034	117	CCTGCCAAGTATGATGAGAT	AGTGTGCGCTGTTGAAGTC

^aALK5, activin receptor-like kinase 5; TGF β 1, transforming growth factor β ; SERPINE1, Serpin family E member 1; ID1, inhibitor of DNA binding 1; ALK1, activin receptor-like kinase 1; COL2A1, type II Collagen α 1; ACAN, aggrecan; ALPL, ALKaline phosphatase; COL10A1, type X Collagen α 1; SPP1, secreted phosphoprotein 1; VEGFA, vascular endothelial growth factor.

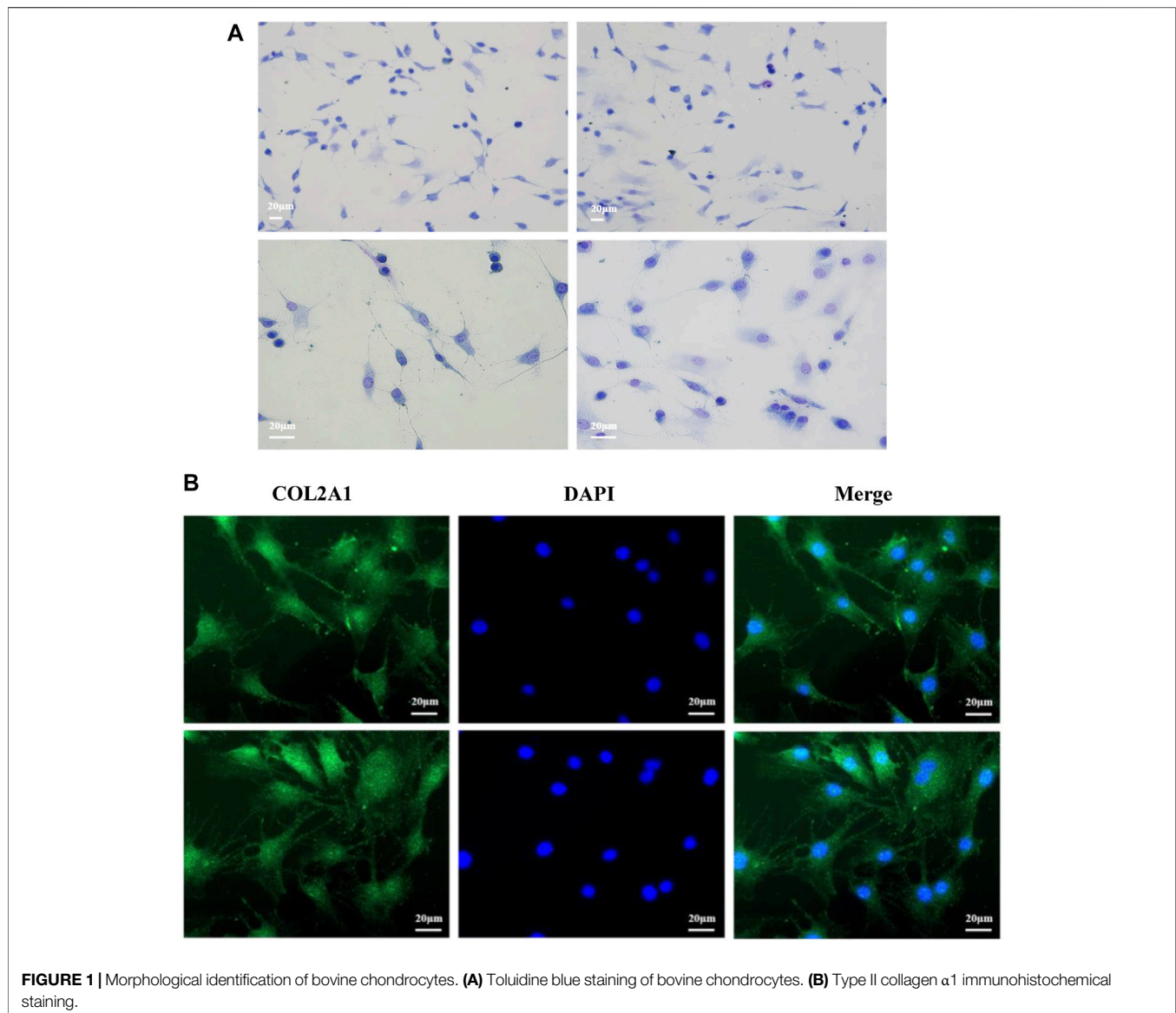


FIGURE 1 | Morphological identification of bovine chondrocytes. **(A)** Toluidine blue staining of bovine chondrocytes. **(B)** Type II collagen $\alpha 1$ immunohistochemical staining.

incubated with 0.25% collagenase type II (1761015, Sigma, United States) at 37°C in 5% CO₂ for 18 h. Cells were collected by passing through a 100-mesh filter and then centrifuged at 400 × g for 10 min with at least three washes. Chondrocytes were then cultured in a 60 mm culture dish (704001, Nest, China) in DMEM/High-glucose (12800017, Gibco, United States) with 10% fetal bovine serum (FB15015, Clark, United States) at 5 × 10⁵ cells/mL in a humidified atmosphere of 5% CO₂ in air at 37°C. The culture medium was replaced regularly every 2 days. When the growth density of the cells reached about 80–90% confluence, cells were passaged using a 0.25% trypsin digestion solution (T1186, Gentihold, China). Cells from second passage were then used for further experiments.

For experiments, cells were treated with a solution of Sr chloride (SrCl₂·6H₂O, V900279, Sigma, United States) dissolved in 0.9% NaCl at different concentrations. In

ruminants, the maximum concentration of Sr used to measure level of Ca absorption from blood is 1 μg/ml (Hyde et al., 2019). Thus, 1 μg/ml was set as the medium-dose group. Doses of 0.1 μg/ml and 10 μg/ml were used as low- and high-dose groups. Cells were cultured for 4 h and 0.9% NaCl was used as a control. To inhibit ALK5 kinase activity, we had another set of cells cultured with the ALK5 inhibitor SB-505124 (HY-13521, MedChemExpress, United States) at a concentration of 10 μM (Vogt et al., 2011; van Caam et al., 2017) for 4 h before treated with 1 μg/ml Sr. 1 μg/ml Sr-treated group served as a control.

Toluidine Blue Staining and Immunofluorescence Staining

Second passage cells on glass coverslips were washed twice using PBS, stained with toluidine blue O (G3660, Solarbio, Beijing,

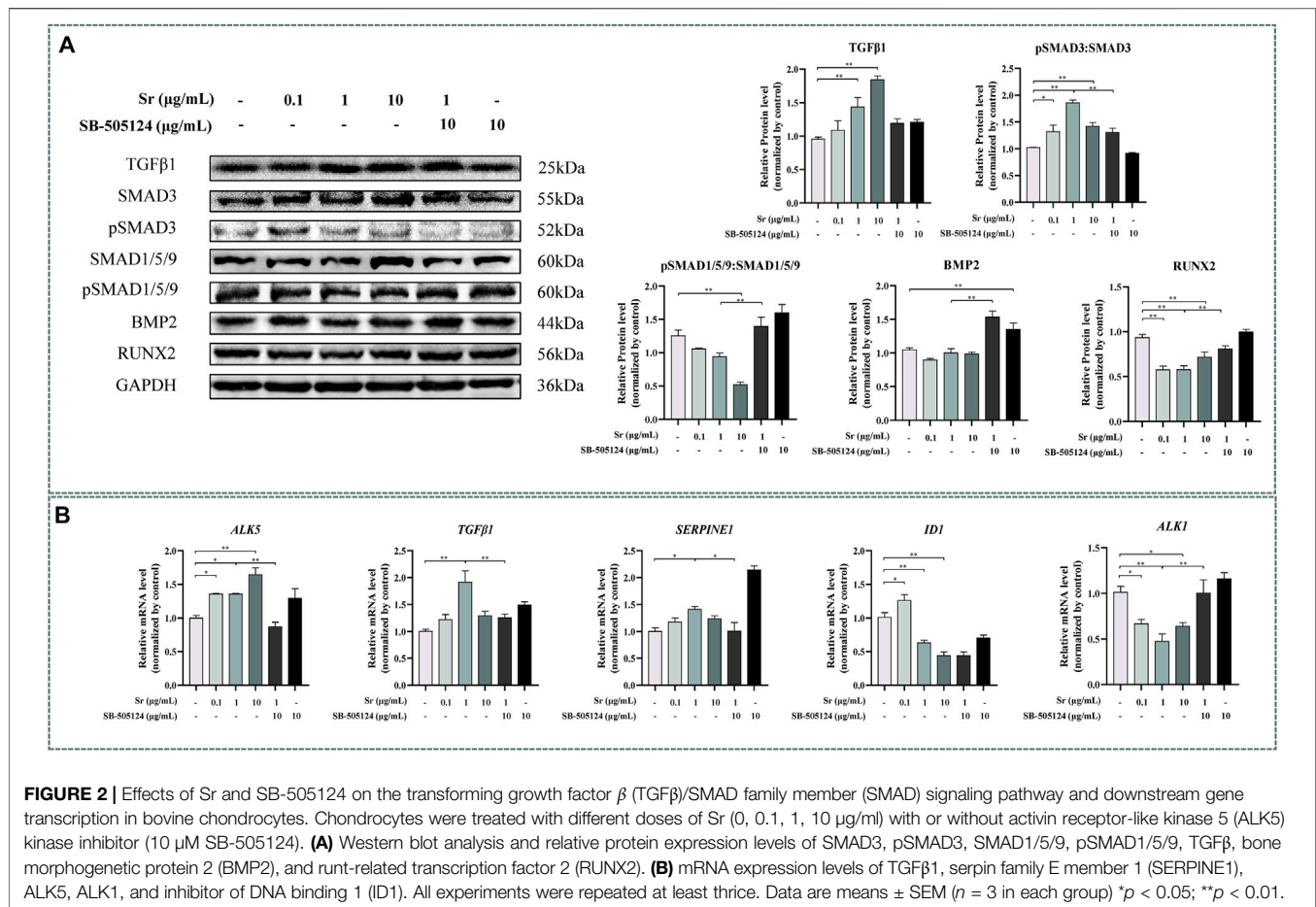


FIGURE 2 | Effects of Sr and SB-505124 on the transforming growth factor β (TGF β)/SMAD family member (SMAD) signaling pathway and downstream gene transcription in bovine chondrocytes. Chondrocytes were treated with different doses of Sr (0, 0.1, 1, 10 μ g/ml) with or without activin receptor-like kinase 5 (ALK5) kinase inhibitor (10 μ M SB-505124). **(A)** Western blot analysis and relative protein expression levels of SMAD3, pSMAD3, SMAD1/5/9, pSMAD1/5/9, TGF β , bone morphogenetic protein 2 (BMP2), and runt-related transcription factor 2 (RUNX2). **(B)** mRNA expression levels of TGF β 1, serpin family E member 1 (SERPINE1), ALK5, ALK1, and inhibitor of DNA binding 1 (ID1). All experiments were repeated at least thrice. Data are means \pm SEM ($n = 3$ in each group) * $p < 0.05$; ** $p < 0.01$.

China) for 5 min. Equal amounts of distilled water were then added and allowed to stand for 15 min. After washing twice with PBS, cells were observed and photographed using a microscope (Carl Zeiss GmbH, Jena, Germany).

Second passage cells on glass coverslips were rinsed three times using PBS and fixed with 4% paraformaldehyde for 30 min. After washing three times with PBS, cells were treated with 0.1% Triton X-100 diluted in PBS for 15 min at 37°C, and blocked with 5% BSA in PBS for 20 min. COL2A1 antibody (COL2A1, AF6528, Beyotime Biotechnology, China) was incubated overnight at 4°C, the glass coverslips rinsed and then incubated with Goat Anti-Rabbit IgG H&L (ab150077, Alexa Fluor 488) for 4 h at 37°C. Cell nuclei were counterstained with DAPI (C1002, Beyotime Biotechnology, China). Lastly, glass coverslips were observed and photographed using a fluorescence microscope (Carl Zeiss GmbH, Jena, Germany).

Total RNA Extraction, Primer Design, and qRT-PCR

Total RNA was extracted using Trizol reagent (15596026, Invitrogen, Carlsbad, United States) following manufacturer's protocols. Concentration and purity of RNA were checked with a NanoDrop 2000C (Thermo Scientific, Waltham, MA, United States). Samples had an optical density ratio at 260/

280 nm > 1.9 and < 2.2 . Reverse transcription of the total RNA was conducted using SuperScript™ RT reagent kit (RR047A, TaKaRa, Japan). All primers used were designed with Primer Premier 6 (Premier Biosoft, United States) based on GenBank data. Primer sequences are listed in **Table 1**. The housekeeping gene *GAPDH* was used as an internal control. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 2 \times M5 HiPer SYBR Premix EsTaq (MF787-01, Meisbio, China).

Protein Extraction and Western Blotting

Total protein was extracted from chondrocytes using the radioimmunoprecipitation assay Lysis Buffer (P0013B, Beyotime Biotechnology, China), and concentration measured with the BCA Protein Assay Kit (P0012, Beyotime Biotechnology, China). Then, 50 μ g protein were separated on a 10% or 8% bisacrylamide gel and transferred to a PVDF membrane (IPVH00010, Millipore, United States). Membranes were blocked with 5% skimmed milk or BSA solution in TBS-T buffer for 2 h, and incubated overnight at 4°C with antibodies including type II Collagen α 1 (COL2A1, 1:1000, AF6528, Beyotime Biotechnology, China), aggrecan (ACAN, 1:500, NB110-6524, Novusbio biologicals, United States), type X Collagen α 1 (COL10A1, 1:500, bs0554R, Bioss biotechnology, China), Osteopontin (OPN, 1:1000, also called SPP1, secreted

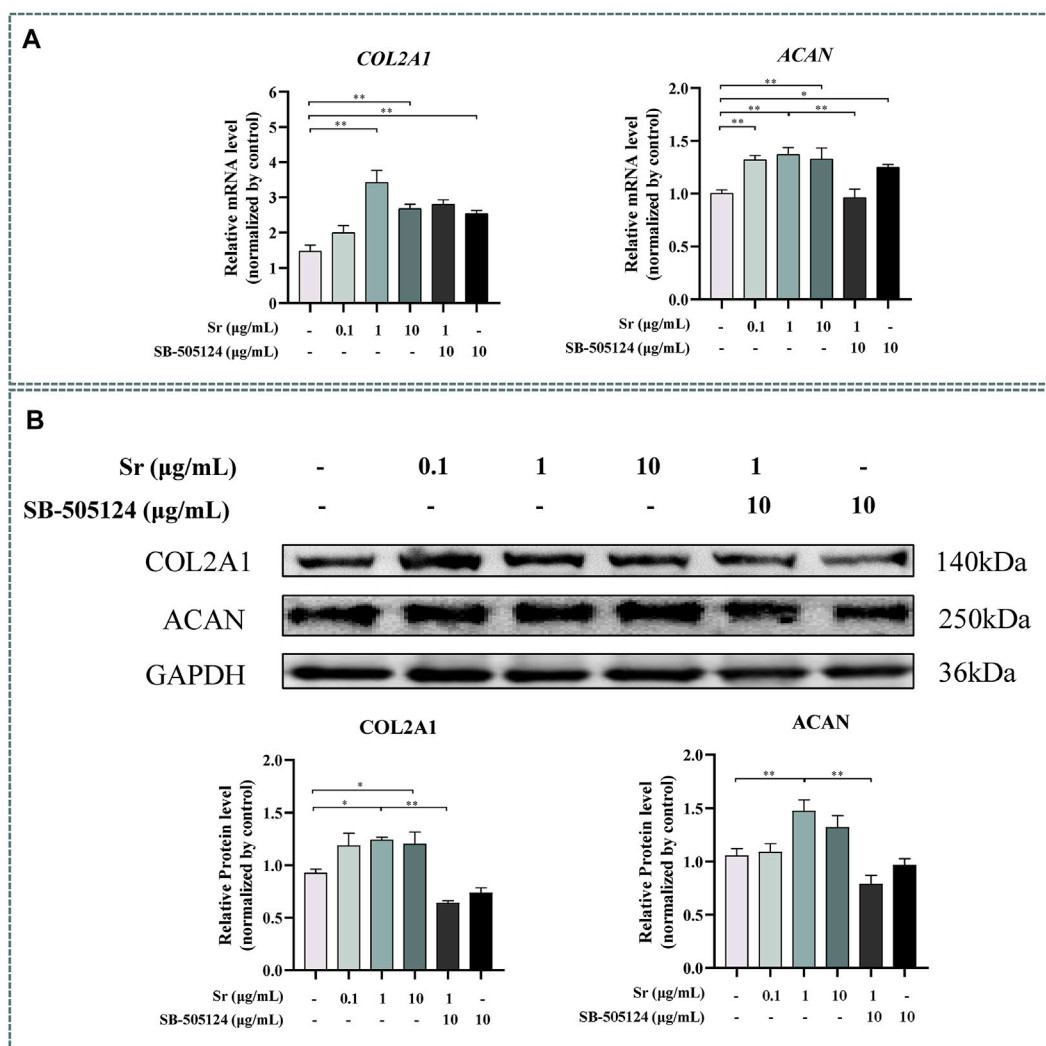


FIGURE 3 | Effects of Sr and SB-505124 on proliferation-related factors in bovine chondrocytes. Chondrocytes were treated with different doses of Sr (0, 0.1, 1, 10 µg/ml) with or without activin receptor-like kinase 5 (ALK5) kinase inhibitor (10 µM SB-505124). **(A)** Relative mRNA expression levels of type II Collagen α1 (COL2A1) and aggrecan (ACAN). **(B)** Western blot and protein expression levels of COL2A1 and ACAN. All experiments were repeated at least thrice. Data are means ± SEM ($n = 3$ in each group) * $p < 0.05$; ** $p < 0.01$.

phosphoprotein 1, bs0019R, Bioss biotechnology, China), vascular endothelial growth factor (VEGFA, 1:1000, NB110-2381ss, Novus Bio biologicals, United States), alkaline phosphatase (ALPL, 1:1000, DF6225, Affinity, United States), SMAD3 (1:500, NB100-56479ss, Novusbio biologicals, United States), pSMAD3 (1:1000, 9520T, Cell Signaling Technology, United States), SMAD1/5/9 (1:1000, AF0614, Affinity, United States), pSMAD1/5/9 (1:1000, 13820T, Cell Signaling Technology, United States) and runt-related transcription factor 2 (RUNX2, 1:1000, AF5189, Affinity, United States). Blots were incubated for 2 h in a horseradish peroxidase (HPR)-conjugated secondary antibody at 25°C. Membranes were detected using a chemiluminescence (ECL) system (ProteinSimple, Santa Clara, CA, United States). Results were analyzed using the ImageJ software (Media Cybernetics, Bethesda, MD, United States).

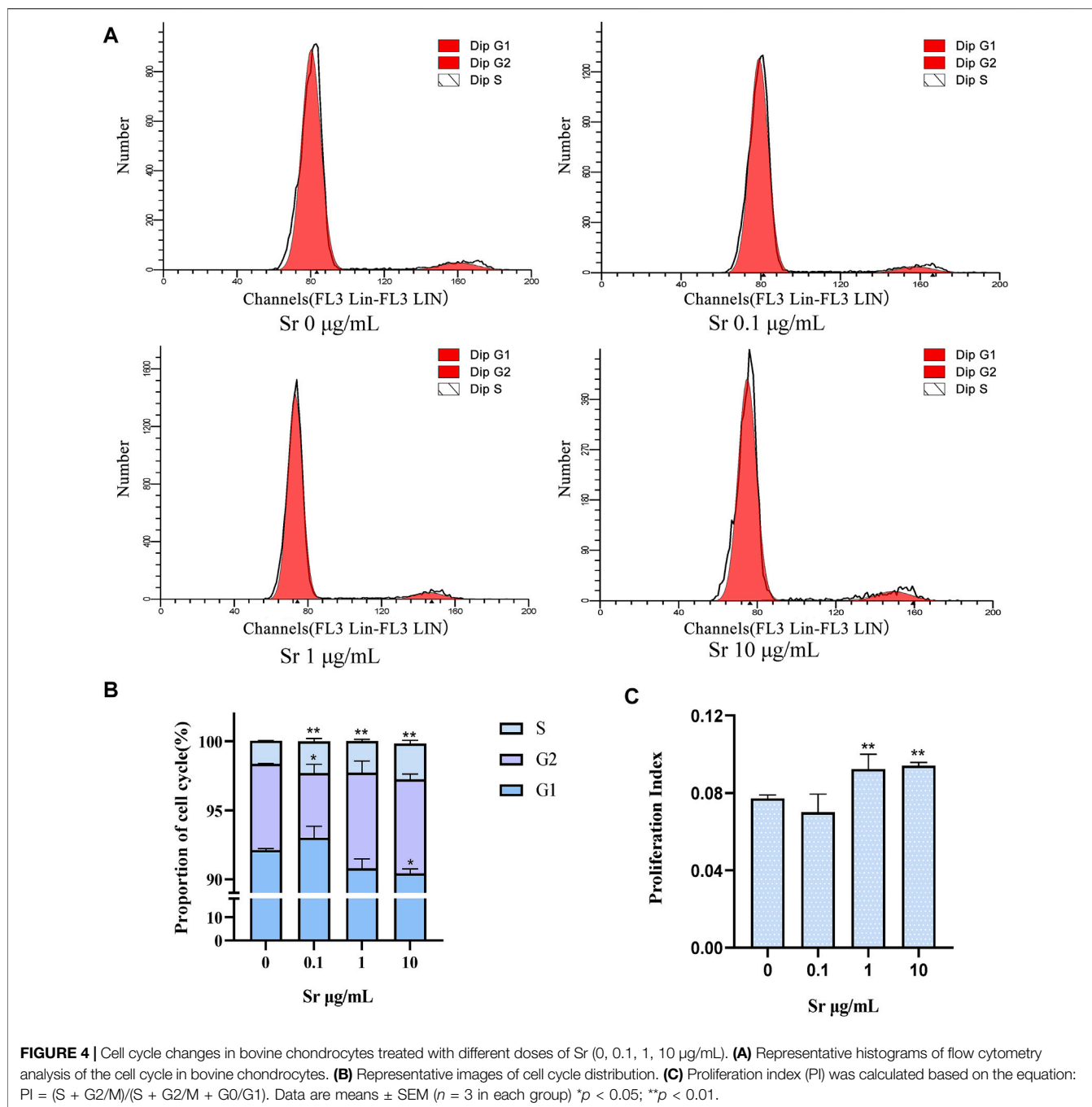
Flow Cytometry

Cells were collected and stained with PI/RNase Staining Buffer (BD pharmingen) to measure cell cycle activity determined by flow cytometry (Coulter-XL). Data were analyzed in ModFit 3.0 (Verity Software House, ME, United States) and the proliferation index (PI) was calculated using the following equations (Ming et al., 2019):

$$PI = (S + G2/M) / (S + G2/M + G0/G1)$$

Coomassie Blue Staining of the Cytoskeleton

Second passage cells in a 35 mm culture dish were cultured at a density of 5.0×10^4 cells/mL. After treatment, cells were washed three times with 0.1% PBS and 1% TritonX-100 was added to each



dish for incubation in a humidified 37°C incubator for 15 min. The dish was washed three times with buffer M (60 mM imidazole, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, pH7.5) to stabilize the cytoskeleton. Cells were then fixed in 3% glutaraldehyde for 30 min and washed three times with 0.1% PBS after fixation, followed by addition of 0.2% Coomassie blue R₂₅₀ for 30 min. Cells were washed with running water and observed and photographed using a microscope (Carl Zeiss GmbH, Jena, Germany).

Statistical Analysis

Each experiment was repeated at least 3 times on consecutive days. The data were analyzed using the GraphPad Prism 7.0 (GraphPad Software Inc., United States) software. All data are reported as means \pm SEM. For data with dose-response effects to Sr, comparisons among groups were performed using one-way ANOVA with Dunnett's multiple comparison test with 0.9% NaCl as the control group. For data with effects of preincubation with SB-505124, comparisons among groups were performed

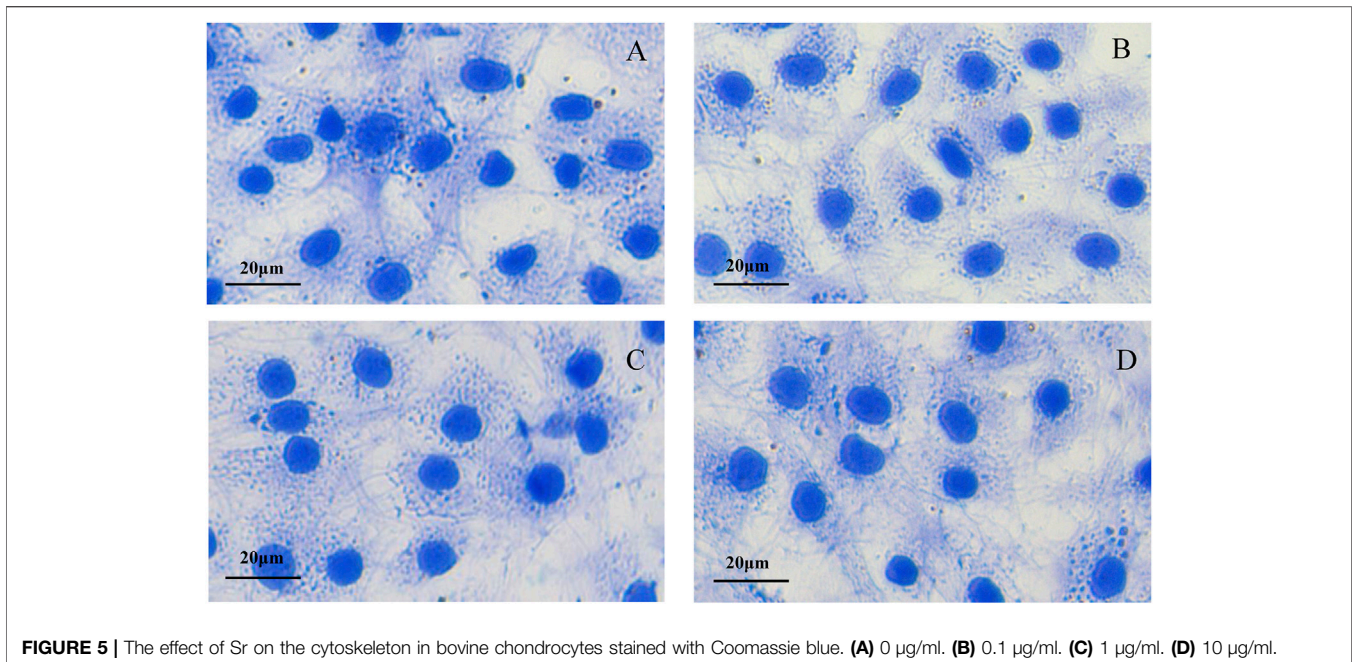


FIGURE 5 | The effect of Sr on the cytoskeleton in bovine chondrocytes stained with Coomassie blue. **(A)** 0 µg/ml. **(B)** 0.1 µg/ml. **(C)** 1 µg/ml. **(D)** 10 µg/ml.

using one-way ANOVA with Dunnett's multiple comparison test with 1 µg/ml Sr as the control. $p < 0.05$ was considered significant.

RESULTS

Morphological Identification of Bovine Chondrocytes

Primary bovine chondrocytes were identified by Toluidine blue staining and type II collagen immunofluorescence staining. Chondrocytes were stained purple by toluidine blue (Figure 1A), and Type II collagen in chondrocytes stained green (type II collagen immunofluorescence). The nuclei stained blue when incubated with DAPI (Figure 1B). Together, these results indicated isolated cells from articular cartilage were chondrocytes.

Strontium Promotes Proliferation and Inhibits Differentiation of Chondrocytes via TGFβ/SMAD3

Exogenous Sr upregulated expression of TGFβ1 at both the transcriptional and protein level (Figure 2). Compared with the control, pSMAD3:SMAD3 ratio was higher (Figure 2A, $p < 0.05$) and the pSMAD1/5/9:SMAD1/5/9 ratio lower ($p < 0.01$) with each dose of Sr. Exogenous Sr did not alter BMP2 ($p = 0.27$ at 0.1 µg/ml; $p = 0.97$ at 1 µg/ml; $p = 0.92$ at 10 µg/ml, Figure 2A). Compared with the control, abundance of the key transcription factor RUNX2, regulated by pSMAD3 and pSMAD1/5/9, was decreased with each dose of Sr (Figure 2A, $p < 0.01$).

As evident from Figure 1B, abundance of the SMAD3-dependent genes *TGFβ1* and *ALK5* increased upon treatment with Sr at all doses

($p < 0.05$). Serpin family E member 1 (*SERPINE1*), another SMAD3-dependent gene, was greater with 1 µg/ml ($p < 0.05$). In contrast to the SMAD3-dependent genes, the abundance of inhibitor of DNA binding 1 (*ID1*) and *ALK1*, both SMAD1/5/9-dependent genes, decreased in all Sr-treated groups compared with the control ($p < 0.05$).

Strontium Promotes Proliferation of Chondrocytes

Both *COL2A1* and *ACAN* were upregulated by treating with Sr and reached the highest level in the 1 µg/ml group ($p < 0.01$) (Figure 3A). The upregulation of *COL2A1* and *ACAN* was also confirmed at the protein level (Figure 3B), with upregulation of *COL2A1* in the 1 and 10 µg/ml groups ($p < 0.05$) while *ACAN* was upregulated in the 1 µg/ml group ($p < 0.01$).

Compared with the control, treatment with Sr led to a remarkable increase in cells at the S-phase from 1.66 to 2.31% in the 0.1 µg/ml group to 2.27 and 2.59% in the 1 µg/ml and 10 µg/ml group (Figures 4A,B). Treatment with 10 µg Sr/mL decreased the number of cells in the G1 phase and 0.1 µg/ml Sr decreased cells in the G2 phase cells (Figures 4A,B). The proliferation index was higher in cells treated with 1 and 10 µg/ml Sr (Figure 4C). Culture with Sr had no effect on the cytoskeleton (Figure 5).

Strontium Inhibits Differentiation of Chondrocytes

Western blotting indicated that Sr treatment downregulated ALPL expression at a concentration of 1 and 10 µg/ml Sr ($p < 0.05$, Figure 6A). Expression of *COL10A1* decreased in the 10 µg/ml treatment group, and expression of *SPP1* decreased in 1 and 10 µg/ml Sr groups ($p < 0.01$, Figure 6A). Compared with the

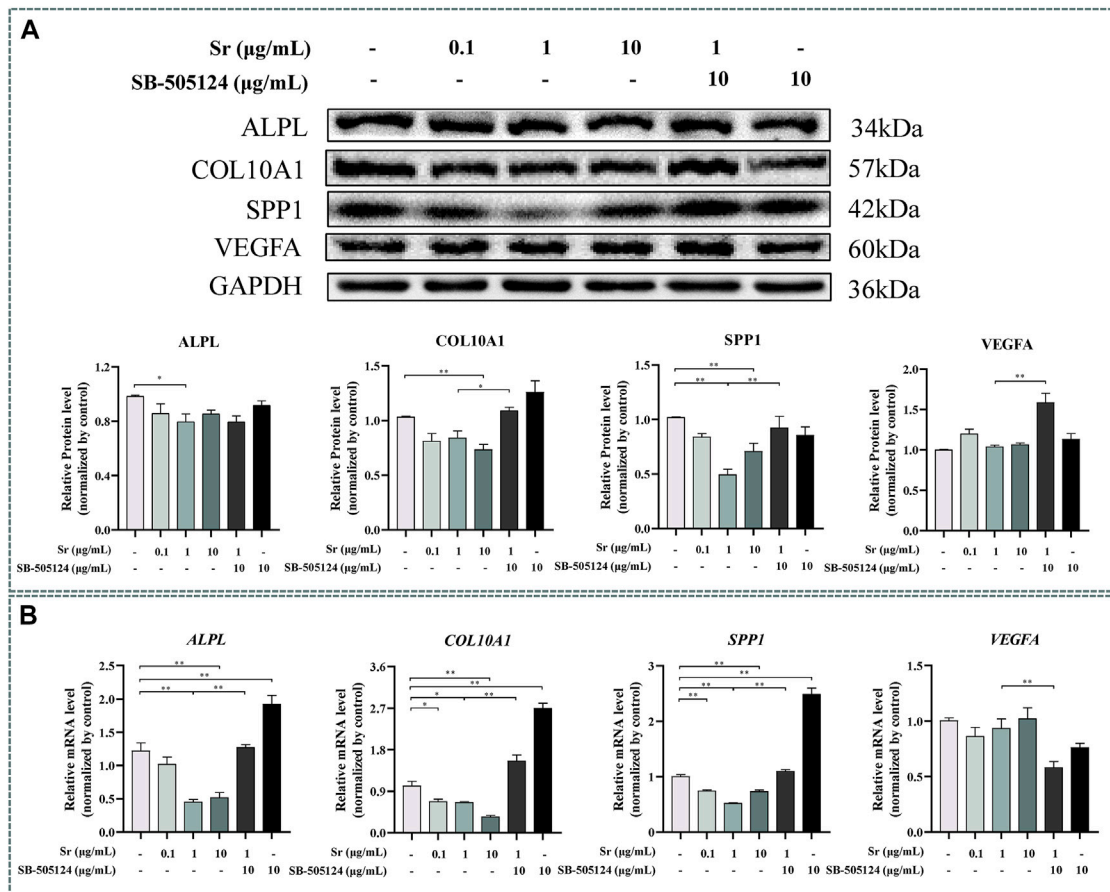


FIGURE 6 | Effects of Sr and SB-505124 on differentiation-related factors in bovine chondrocytes. Chondrocytes were treated with different doses of Sr (0, 0.1, 1, 10 µg/ml) with or without activin receptor-like kinase 5 (ALK5) kinase inhibitor (10 µM SB-505124). **(A)** Western blot and protein expression levels of secreted phosphoprotein 1 (SPP1), alkaline phosphatase (ALPL), vascular endothelial growth factor (VEGFA), and type X Collagen α1 (COL10A1). **(B)** mRNA expression levels of SPP1, ALPL, VEGFA, and COL10A1. All experiments were repeated at least thrice. Data are means ± SEM ($n = 3$ in each group) * $p < 0.05$; ** $p < 0.01$.

control, there was no effect of Sr on the expression of VEGFA. However, qPCR results revealed a marked downregulation of *ALPL* upon treatment with Sr at 1 µg/ml and 10 µg/ml ($p < 0.01$, **Figure 6B**). Both *COL10A1* and *SPP1* decreased in all Sr-treated groups ($p < 0.05$, **Figure 6B**). Dose of Sr had no effect on *VEGFA* expression.

Strontium-Induced SMAD3 Phosphorylation

Pre-incubation with SB-505124 inhibited Sr-induced SMAD3 phosphorylation and promoted Sr-induced SMAD1/5/9 phosphorylation ($p < 0.01$, **Figure 2A**). SB-505124 inhibited mRNA expression of the SMAD3-dependent genes *TGFβ1*, *ALK5*, and *SERPINE1* ($p < 0.05$, **Figure 2B**). Although there was no significant change in *ID1* ($p = 0.48$), SB-505124 increased mRNA expression of *ALK1* ($p < 0.01$, **Figure 2B**).

Expression of *ACAN* mRNA decreased ($p < 0.01$, **Figure 3A**) while protein abundance of *ACAN* and *COL2A1* decreased at the protein level when cells were pre-incubated with SB-505124 ($p < 0.01$, **Figure 3B**). Abundance of *ALPL*, *COL10A1*, and *SPP1*

mRNA all increased ($p < 0.01$, **Figure 6B**) while protein expression of *COL10A1* and *SPP1* also increased ($p < 0.05$, **Figure 6A**).

DISCUSSION

Some studies have reported that Sr absorption could be used as a surrogate indicator for evaluating Ca absorption in the gastrointestinal tract in dairy cows and sheep (Hyde and Fraser, 2014; Hyde et al., 2019). However, the last few decades have witnessed studies on the effect of Sr in the cartilage and chondrocyte. For instance, Sr can reduce cartilage degeneration and promote ECM production in the ovariectomized rats (Mierzwa et al., 2017). Strontium gluconate was increased mRNA expression of *COL2A1* and *ACAN* in the osteoarthritic rat model (Hu et al., 2020). Thus, these studies provided some evidence for a role of Sr on the regulation of proliferation and differentiation of the cartilage. Unlike rodents, the role of Sr and the underlying molecular mechanisms on proliferation and differentiation of

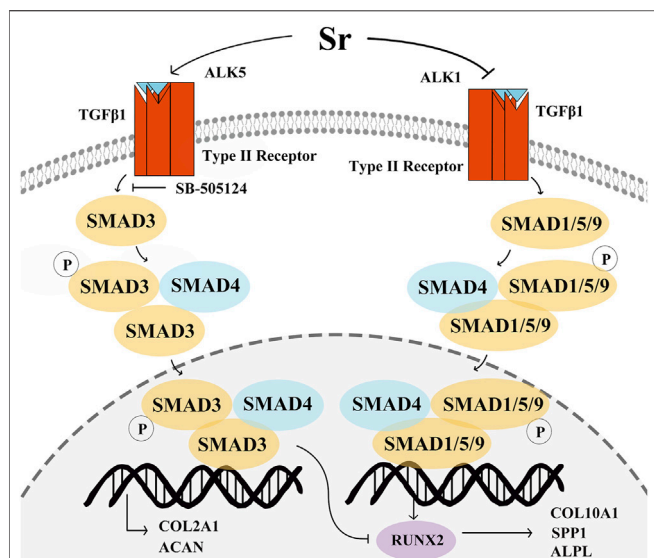


FIGURE 7 | Schematic of the proposed mechanism whereby Sr affects proliferation and differentiation of bovine chondrocytes via the transforming growth factor β (TGF β) signaling pathway. Sr shifts the signaling of TGF β 1 to SMAD family member 3 (SMAD3) by promoting activin receptor-like kinase 5 (ALK5) expression and inhibiting ALK1 expression. Phosphorylation of SMAD3 and SMAD1/5/9 after binding with SMAD4 transfers them into the nucleus to regulate gene transcription. An increase in pSMAD3 promotes the expression of type II Collagen α 1 (COL2A1) and aggrecan (ACAN). pSMAD1/5/9 induces type X Collagen α 1 (COL10A1), secreted phosphoprotein 1 (SPP1), vascular endothelial growth factor (VEGFA), and alkaline phosphatase (ALPL) by stimulating the function of runt-related transcription factor 2 (RUNX2). The increase in the pSMAD3 can inhibit the function of RUNX2 to decrease the COL10A1, SPP1, and ALPL expression.

chondrocytes in ruminants are not well known. Further, whether Sr regulates chondrocyte proliferation and differentiation *via* the TGF β pathway remains unclear. The present study demonstrated that Sr promoted TGF β 1/ALK5-induced SMAD3 phosphorylation and inhibited ALK1-induced SMAD1/5/9 phosphorylation. Sr promoted the expression of the proliferation-responsive factors COL2A1 and ACAN while inhibiting the expression of differentiation-responsive factors COL10A1, SPP1, and ALPL both at the mRNA and protein levels.

In non-ruminants, TGF β 1 is well-known to regulate proliferation and differentiation of chondrocytes (Wu et al., 2016). It plays an anti-hypertrophic role *via* the most classical ALK5/SMAD2/3-dependent pathway (Thielen et al., 2019), and several studies have reported that TGF β 1 also induces the SMAD1/5/9-dependent pathway *via* ALK1 (Chen et al., 2012; Charlier et al., 2019; Thielen et al., 2019). These two pathways have antagonistic functions in the chondrocytes (Finsson et al., 2008). For instance, a study in human chondrocytes reported that TGF β 1 could induce SMAD2 phosphorylation *via* ALK5 and SMAD1/5/9 phosphorylation *via* ALK1 (Finsson et al., 2010). It was also demonstrated that phosphorylation of SMAD2 increased the expression of COL2A1. Zhang et al. (2017) working with newborn mice reported that TGF β regulated SMAD1/5/9 phosphorylation

via the ALK1 in pulmonary artery smooth muscle cells and fibroblasts.

The significant increase of TGF β 1 levels in response to 1 μ g/ml and 10 μ g/ml Sr provided direct evidence that this mineral can affect TGF β signaling. Furthermore, the present study determined that Sr, at a low concentration (i.e., 0.1 μ g/ml), altered the pSMAD3:SMAD3 and the pSMAD1/5/9:SMAD1/5/9 ratio in a way that underscores its potential regulatory role of the TGF β 1 pathway. Such an effect is important in the context of biological responses that this mineral can induced in the bovine. For instance, Sr-containing α -calcium sulfate hemihydrate promoted osteogenic differentiation through TGF β 1-induced SMAD2/3 phosphorylation (Liu et al., 2019). In addition, in one of our previous studies we demonstrated that Sr can activate TGF β 1 signaling in rat chondrocytes (Kong et al., 2018). Overall, the results of the present study were consistent with the functional link between Sr and TGF β signaling.

Existing studies have demonstrated that SMAD3 can be regulated by TGF β through ALK5 (Li et al., 2005). This mechanism was confirmed in the present study when pre-treating chondrocytes with SB-505124 (Byfield et al., 2004; Vogt et al., 2011), the inhibitor of ALK5-mediated SMAD3 phosphorylation, inhibited SMAD3 phosphorylation in the co-treated group. Hellingman et al. (2011) reported that blocking SMAD2/3 with SB-505124 decreased abundance of COL2A1, which our results confirmed that COL2A1 and ACAN significantly decreased in the co-treatment group. In non-ruminants, BMP2, 4, 6, 7, and 9 are well-known to regulate SMAD1/5/9 *via* ALK1 (Li et al., 2004; Dexheimer et al., 2016). In addition, TGF β also regulates SMAD1/5/9 *via* both ALK1 and ALK5 (Finsson et al., 2008; van Caam et al., 2017). Taking all these into account, although the present study did not detect effects of Sr on BMP2, any potential effects on BMP4, 6, 7, and 9 on the SMAD1/5/9 pathway cannot be excluded. Additional studies are needed to identify the mechanism whereby Sr can affect this pathway.

Previous studies in non-ruminants have reported that SMAD3-dependent genes such as TGF β 1, SERPINE1, and ALK5 (van Caam et al., 2015) are upregulated in response to Sr. *ID1* and *ALK1* are well-known SMAD1/5/9-dependent genes in non-ruminants (Ehrichtiou et al., 2010). Thus, the downregulation of *ID1* and *ALK1* at doses of 1 and 10 μ g/ml Sr were in line with results demonstrating that Sr activated the SMAD3-dependent pathway while inhibiting the SMAD1/5/9-dependent pathway, the latter being a response already demonstrated in murine chondrocytes (Ehrichtiou et al., 2010).

The differentiation and maturation of chondrocytes in non-ruminants is positively regulated, at least *in vitro*, by RUNX2 (Komori, 2018). This transcription factor promotes the expression of hypertrophy makers such as COL10A1, SPP1, VEGFA, and ALPL (Komori, 2017). Several studies reported a closed relationship between RUNX2 and SMAD proteins, and demonstrated that SMAD1/5/9 promotes chondrocyte maturation by stimulating the RUNX2 function, with SMAD3 serving to counteract RUNX2 (Wu et al., 2016; Yu et al., 2019). In the current study, the downregulation of RUNX2 in response to

Sr was consistent with the downregulation of COL10A1 and SPP1. From these responses we speculate that SMAD3 and SMAD1/5/9 could at least partly regulate chondrocyte maturation *via* RUNX2. Support for this idea arises from data indicating that macrophages stimulated by 10 ng/ml IL-4 induced hypertrophy of human chondrocytes by promoting COL10A1 and RUNX2 (Ferrao Blanco et al., 2021). A role for RUNX2 on controlling hypertrophy of the tibial growth plate also was demonstrated in broilers (Wang et al., 2021). Overall, the present results were consistent with previous data.

The structural proteins COL2A1 and ACAN are upregulated in the proliferating zone during cartilage maturation, and when chondrocytes undergo hypertrophy, the proteins VEGFA, SPP1, COL10A1, and ALPL are secreted (Kronenberg, 2003; Kozhemyakina et al., 2015; Charlier et al., 2019; Vimalraj, 2020). In our previous study with rat primary chondrocytes there was a dose-dependent upregulation in COL2A1 with 1, 3 and 5 mM Sr (Wang et al., 2013). The fact that Sr promoted the expression of COL2A1 and ACAN at both mRNA and protein levels along with an increase in the PI suggested that Sr could promote chondrocyte proliferation in the bovine. Similarly, these results were also in agreement with data in human primary chondrocytes demonstrating that Sr chondroitin sulfate markedly upregulated the expression of COL2A1 and ACAN (Ma et al., 2017). The TGF β co-receptor Cripto promoted COL10A1 by inducing SMAD1/5/9 signaling in ATDC5 cells and immortalized C28/12 human chondrocytes (Garcia de Vinuesa et al., 2021). A role for ALPL in the maturation and mineralization as well as the inhibitory effect of Sr on SPP1, COL10A1, and ALPL was reported in murine chondrocytes (Ehriouchi et al., 2020). Thus, together, available data suggest a mechanistic function for the systemic supply of Sr on fundamental aspects of chondrocyte development (Figure 7).

Similar to non-ruminants, Sr promotes SMAD3 phosphorylation and transcription of its downstream genes *via* the TGF β 1/ALK5 pathway, including COL2A1 and ACAN, both of which are key factors in the proliferation of chondrocytes. Differentiation of chondrocytes is controlled by Sr *via* decreasing the ALK1-induced SMAD1/5/9 phosphorylation and transcription of SPP1, COL10A1, and ALPL, all of which are key factors in differentiation. Overall, Sr activates TGF β 1-

signaling towards phosphorylation of SMAD3 and, as such, systemic availability of this mineral can directly affect chondrocyte biology in dairy cattle.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare and Research Ethics Committee at Northwest A&F University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SL and BS contributed to conception and design of the study, performed the experiments and wrote original draft. JL and QJ contributed to manuscript revision and editing. YY performed immunocytochemistry experiments. YK, PT, and FZ performed the statistical analysis. CZ and XZ designed the study. JW edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.925302/full#supplementary-material>

REFERENCES

- Blaney Davidson, E. N., Remst, D. F., Vitters, E. L., van Beuningen, H. M., Blom, A. B., Goumans, M. J., et al. (2010). Increase in ALK1/ALK5 Ratio as a Cause for Elevated MMP-13 Expression in Osteoarthritis in Humans and Mice. *J. Immunol.* 182 (12), 7937–7945. doi:10.4049/jimmunol.109006810.4049/jimmunol.0803991
- Charlier, E., Deroyer, C., Ciregia, F., Malaise, O., Neuville, S., Plener, Z., et al. (2019). Chondrocyte Dedifferentiation and Osteoarthritis (OA). *Biochem. Pharmacol.* 165, 49–65. doi:10.1016/j.bcp.2019.02.036
- Chen, G., Deng, C., and Li, Y. P. (2012). TGF- β and BMP Signaling in Osteoblast Differentiation and Bone Formation. *Int. J. Biol. Sci.* 8 (2), 272–288. doi:10.7150/ijbs.2929
- DaCosta Byfield, S., Major, C., Laping, N. J., and Roberts, A. B. (2004). SB-505124 Is a Selective Inhibitor of Transforming Growth Factor-Beta Type I Receptors

ALK4, ALK5, and ALK7. *Mol. Pharmacol.* 65 (3), 744–752. doi:10.1124/mol.65.3.744

- Dexheimer, V., Gabler, J., Bomans, K., Sims, T., Omlor, G., and Richter, W. (2016). Differential Expression of TGF- β Superfamily Members and Role of Smad1/5/9-Signalling in Chondral versus Endochondral Chondrocyte Differentiation. *Sci. Rep.* 6, 36655. doi:10.1038/srep36655
- Ehriouchi, D., Bernabei, I., Chobaz, V., Castelblanco, M., Hügle, T., So, A., et al. (2020). CD11b Signaling Prevents Chondrocyte Mineralization and Attenuates the Severity of Osteoarthritis. *Front. Cell Dev. Biol.* 8, 611757. doi:10.3389/fcell.2020.611757
- Ferrao Blanco, M. N., Bastiaansen-Jenniskens, Y. M., Chambers, M. G., Pittsillides, A. A., Narcisi, R., and van Osch, G. J. V. M. (2021). Effect of Inflammatory Signaling on Human Articular Chondrocyte Hypertrophy: Potential Involvement of Tissue Repair Macrophages. *Cartilage* 13, 168S–174S. doi:10.1177/19476035211021907

- Finsson, K. W., Parker, W. L., Chi, Y., Hoemann, C. D., Goldring, M. B., Antoniou, J., et al. (2010). Endoglin Differentially Regulates TGF- β -Induced Smad2/3 and Smad1/5 Signaling and its Expression Correlates with Extracellular Matrix Production and Cellular Differentiation State in Human Chondrocytes. *Osteoarthr. Cartil.* 18 (11), 1518–1527. doi:10.1016/j.joca.2010.09.002
- Finsson, K. W., Parker, W. L., ten Dijke, P., Thorikay, M., and Philip, A. (2008). ALK1 Opposes ALK5/Smad3 Signaling and Expression of Extracellular Matrix Components in Human Chondrocytes. *J. Bone Min. Res.* 23 (6), 896–906. doi:10.1359/jbmr.080209
- García de Vinuesa, A., Sanchez-Duffhues, G., Blaney-Davidson, E., van Caam, A., Lodder, K., Ramos, Y., et al. (2021). Cripto Favors Chondrocyte Hypertrophy via TGF- β SMAD1/5 Signaling during Development of Osteoarthritis. *J. Pathol.* 255 (3), 330–342. doi:10.1002/path.5774
- Hellingman, C. A., Davidson, E. N., Koevoet, W., Vitters, E. L., van den Berg, W. B., van Osch, G. J., et al. (2011). Smad Signaling Determines Chondrogenic Differentiation of Bone-Marrow-Derived Mesenchymal Stem Cells: Inhibition of Smad1/5/8P Prevents Terminal Differentiation and Calcification. *Tissue Eng. Part A* 17 (7-8), 1157–1167. doi:10.1089/ten.TEA.2010.0043
- Hernández-Castellano, L. E., Hernandez, L. L., and Bruckmaier, R. M. (2020). Review: Endocrine Pathways to Regulate Calcium Homeostasis Around Parturition and the Prevention of Hypocalcemia in Periparturient Dairy Cows. *Animal* 14 (2), 330–338. doi:10.1017/S1751731119001605
- Hu, P., Du, J., Zhang, S., Wang, T., Li, J., Chen, G., et al. (2020). Oral Administration of Strontium Gluconate Effectively Reduces Articular Cartilage Degeneration through Enhanced Anabolic Activity of Chondrocytes and Chondrogenetic Differentiation of Mesenchymal Stromal Cells. *Biol. Trace Elem. Res.* 193 (2), 422–433. doi:10.1007/s12011-019-01711-9
- Hyde, M. L., and Fraser, D. R. (2014). *In Vivo* measurement of the Absorption of Strontium in the Rumen and Small Intestine of Sheep as an Index of Calcium Absorption Capacity. *Br. J. Nutr.* 112 (5), 718–724. doi:10.1017/S0007114514001500
- Hyde, M. L., Wilkens, M. R., and Fraser, D. R. (2019). *In Vivo* measurement of Strontium Absorption from the Rumen of Dairy Cows as an Index of Calcium Absorption Capacity. *J. Dairy Sci.* 102 (6), 5699–5705. doi:10.3168/jds.2018-16052
- Khan, A. H., Rohra, D. K., Saghir, S. A., Udani, S. K., Wood, R. J., and Jabbar, A. (2013). No Change in Calcium Absorption in Adult Pakistani Population before and after Vitamin D Administration Using Strontium as Surrogate. *Osteoporos. Int.* 24 (3), 1057–1062. doi:10.1007/s00198-012-2007-9
- Komori, T. (2017). Roles of Runx2 in Skeletal Development. *Adv. Exp. Med. Biol.* 962, 83–93. doi:10.1007/978-981-10-3233-2_6
- Komori, T. (2018). Runx2, an Inducer of Osteoblast and Chondrocyte Differentiation. *Histochem. Cell Biol.* 149 (4), 313–323. doi:10.1007/s00418-018-1640-6
- Kong, Y., Guo, Y., Zhang, J., Zhao, B., and Wang, J. (2018). Strontium Promotes Transforming Growth Factors β 1 and β 2 Expression in Rat Chondrocytes Cultured *In Vitro*. *Biol. Trace Elem. Res.* 184 (2), 450–455. doi:10.1007/s12011-017-1208-7
- Kozhemyakina, E., Lassar, A. B., and Zelzer, E. (2015). A Pathway to Bone: Signaling Molecules and Transcription Factors Involved in Chondrocyte Development and Maturation. *Development* 142 (5), 817–831. doi:10.1242/dev.105536
- Kronenberg, H. M. (2003). Developmental Regulation of the Growth Plate. *Nature* 423 (6937), 332–336. doi:10.1038/nature01657
- Li, T.-F., Darowish, M., Zuscik, M. J., Chen, D., Schwarz, E. M., Rosier, R. N., et al. (2004). Smad3-Deficient Chondrocytes Have Enhanced BMP Signaling and Accelerated Differentiation. *J. Bone Min. Res.* 21, 4–16. doi:10.1359/JBMR.050911
- Li, T. F., O'Keefe, R. J., and Chen, D. (2005). TGF-beta Signaling in Chondrocytes. *Front. Biosci.* 10, 681–688. doi:10.2741/1563
- Liu, Z., Yu, Z., Chang, H., Wang, Y., Xiang, H., Zhang, X., et al. (2019). Strontium-containing α -calcium Sulfate Hemihydrate Promotes Bone Repair via the TGF- β /Smad Signaling Pathway. *Mol. Med. Rep.* 20 (4), 3555–3564. doi:10.3892/mmr.2019.10592
- Ma, F. B., Liu, N., Hu, N., Wen, C. Y., and Tang, B. (2017). Synthesis of Strontium Chondroitin Sulfate and the Evaluation of its Capability to Attenuate Osteoarthritis. *Carbohydr. Polym.* 170, 217–225. doi:10.1016/j.carbpol.2017.04.067
- Madej, W., van Caam, A., Blaney Davidson, E., Buma, P., and van der Kraan, P. M. (2016). Unloading Results in Rapid Loss of TGF β Signaling in Articular Cartilage: Role of Loading-Induced TGF β Signaling in Maintenance of Articular Chondrocyte Phenotype? *Osteoarthr. Cartil.* 24, 1807–1815. doi:10.1016/j.joca.2016.05.018
- Mierzwa, A. G. H., Campos, J. F., Jesus, M. F., Nader, H. B., Lazaretti-Castro, M., and Reginato, R. D. (2017). Different Doses of Strontium Ranelate and Mechanical Vibration Modulate Distinct Responses in the Articular Cartilage of Ovariectomized Rats. *Osteoarthr. Cartil.* 25 (7), 1179–1188. doi:10.1016/j.joca.2017.02.793
- Milsom, S., Ibbertson, K., Hannan, S., Shaw, D., and Pybus, J. (1987). Simple Test of Intestinal Calcium Absorption Measured by Stable Strontium. *Br. Med. J. Clin. Res. Ed.* 295 (6592), 231–234. doi:10.1136/bmj.295.6592.231
- Ming, J., Wu, S., You, T., Wang, X., Yu, C., Luo, P., et al. (2019). Histone Deacetylation in the Promoter of P16 Is Involved in Fluoride-Induced Human Osteoblast Activation via the Inhibition of Sp1 Binding. *Biol. Trace Elem. Res.* 188 (2), 373–383. doi:10.1007/s12011-018-1413-z
- Pilmame, M., Salma-Ancane, K., Loca, D., Locs, J., and Berzina-Cimdina, L. (2017). Strontium and Strontium Ranelate: Historical Review of Some of Their Functions. *Mater. Sci. Eng. C Mater. Biol. Appl.* 78, 1222–1230. doi:10.1016/j.msec.2017.05.042
- Pors Nielsen, S. (2004). The Biological Role of Strontium. *Bone* 35 (3), 583–588. doi:10.1016/j.bone.2004.04.026
- Querido, W., Rossi, A. L., and Farina, M. (2016). The Effects of Strontium on Bone Mineral: A Review on Current Knowledge and Microanalytical Approaches. *Micron* 80, 122–134. doi:10.1016/j.micron.2015.10.006
- Thielen, N. G. M., van der Kraan, P. M., and van Caam, A. P. M. (2019). TGF β /BMP Signaling Pathway in Cartilage Homeostasis. *Cells* 8 (9), 969. doi:10.3390/cells8090969
- van Caam, A., Blaney Davidson, E., Garcia de Vinuesa, A., van Geffen, E., van den Berg, W., Goumans, M. J., et al. (2015). The High Affinity ALK1-Ligand BMP9 Induces a Hypertrophy-like State in Chondrocytes that Is Antagonized by TGF β 1. *Osteoarthr. Cartil.* 23 (6), 985–995. doi:10.1016/j.joca.2015.02.007
- van Caam, A., Madej, W., Garcia de Vinuesa, A., Goumans, M. J., ten Dijke, P., Blaney Davidson, E., et al. (2017). TGF β 1-induced SMAD2/3 and SMAD1/5 Phosphorylation Are Both ALK5-kinase-dependent in Primary Chondrocytes and Mediated by TAK1 Kinase Activity. *Arthritis Res. Ther.* 19 (1), 112. doi:10.1186/s13075-017-1302-4
- Vimalraj, S. (2020). Alkaline Phosphatase: Structure, Expression and its Function in Bone Mineralization. *Gene* 754, 144855. doi:10.1016/j.gene.2020.144855
- Vogt, J., Traynor, R., and Sapkota, G. P. (2011). The Specificities of Small Molecule Inhibitors of the TGF β and BMP Pathways. *Cell. Signal.* 23 (11), 1831–1842. doi:10.1016/j.cellsig.2011.06.019
- Wang, C. Y., Xia, W. H., Wang, L., and Wang, Z. Y. (2021). Manganese Deficiency Induces Avian Tibial Dyschondroplasia by Inhibiting Chondrocyte Proliferation and Differentiation. *Res. Vet. Sci.* 140, 164–170. doi:10.1016/j.rvsc.2021.08.018
- Wang, J., Zhu, X., Liu, L., Shi, X., Yin, L., Zhang, Y., et al. (2013). Effects of Strontium on Collagen Content and Expression of Related Genes in Rat Chondrocytes Cultured *In Vitro*. *Biol. Trace Elem. Res.* 153 (1-3), 212–219. doi:10.1007/s12011-013-9640-9
- Woods, S., Humphreys, P. A., Bates, N., Richardson, S. A., Kuba, S. Y., Brooks, I. R., et al. (2021). Regulation of TGF β Signaling by TRPV4 in Chondrocytes. *Cells* 10 (4), 726. doi:10.3390/cells10040726
- Wu, M., Chen, G., and Li, Y. P. (2016). TGF- β and BMP Signaling in Osteoblast, Skeletal Development, and Bone Formation, Homeostasis and Disease. *Bone Res.* 4, 16009. doi:10.1038/boneres.2016.9
- Yu, X., Shen, G., Ren, H., Zhang, Z., Shang, Q., Zhao, W., et al. (2019). Tgfb-Induced Factor Homeobox 2 Blocks Osteoblastic Differentiation through Targeting pSmad3/HDAC4/H4ac/Runx2 axis. *J. Cell. Physiol.* 234 (11), 21284–21293. doi:10.1002/jcp.28733

- Zhang, H., Du, L., Zhong, Y., Flanders, K. C., and Roberts, J. D. (2017). Transforming Growth Factor- β Stimulates Smad1/5 Signaling in Pulmonary Artery Smooth Muscle Cells and Fibroblasts of the Newborn Mouse through ALK1. *Am. J. Physiol. Lung Cell Mol. Physiol.* 313 (3), L615–L627. doi:10.1152/ajplung.00079.2017
- Zhu, Y., Hu, C., Zheng, P., Miao, L., Yan, X., Li, H., et al. (2016). Ginsenoside Rb1 Alleviates Aluminum Chloride-Induced Rat Osteoblasts Dysfunction. *Toxicol.* 368–369, 183–188. doi:10.1016/j.tox.2016.07.014

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