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Sinomenine can promote the proliferation and differentiation of osteoblasts by regulating the Akt/Runx2 signaling pathway in MC3T3-E1 cells

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This study aimed to explore the effect of sinomenine on the proliferation and differentiation of MC3T3-E1 cells and the related mechanism. Mouse preosteoblastic cell line MC3T3-E1 cells were divided into four groups: control group, treatment of sinomenine with the concentrations of 100, 500 or 1000 μM. The proliferation and apoptosis of the cells were determined by MTT assay at 0, 24, 48 and 72 h, and flow cytometry method was used to determine the effect of sinomenine on the apoptosis of MC3T3-E1 cells at 72 h; furthermore, after 72 h, the cell culture supernatants were collected, and the levels of alkaline phosphatase and osteocalcin were examined by enzyme-linked immunosorbent assay (ELISA); next, cells were collected, and the expression of type I collagen (COL1A1), osteopontin (OPN), protein kinase B (Akt), runt-related transcription factor 2 (Runx2), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were examined by RT-qPCR and Western Blot methods. It was observed that treatment of 500 and 1000 μM sinomenine has induced significant increase in the proliferation and decrease in the apoptosis of MC3T3-E1 cells; furthermore, sinomenine also lead to increased secretion of osteocalcin and alkaline phosphatase in MC3T3-E1 cells; finally, sinomenine induced marked increase in the expression of type I collagen, osteopontin and also induced the activation of the Akt/Runx2 signaling pathway. To sum up, we observed that sinomenine may promote the proliferation and differentiation of MC3T3-E1 cells via activating the Akt/Runx2 signaling pathway.

1. Introduction

Osteoporosis is a frequently occurring bone disease, and most probably one of the most common reasons for the incidence of bone fractures among the elderlies (Morita et al. 2005). Osteoporosis causing large finical burdens to the public healthcare system (Strom et al. 2011; Vandenbroucke et al. 2017). Based on results of previous studies, it has been widely accepted that aberrances in the proliferation and differentiation of osteoblasts were the mains reasons of osteoporosis (Li et al. 2017; Yu et al. 2012). Therefore, to investigate the effects of potential anti-osteoporosis medication on the proliferation and differentiation of osteoblasts is of great importance for the development of novel anti-osteoporosis therapies.

Sinomenine (Fig. 1A) is the main component of *Sinomenium acutum*, which is a the traditional Chinese medicine (Wang et al. 2016). It has anti-inflammatory (Chen et al. 2011), anti-antioxidant (Qin et al. 2016) and anti-cancer (Song et al. 2015) effects. The therapeutic roles of sinomenine in bone and cartilage diseases have also been discussed previously. Yao et al. (2017) reported that sinomenine can exert anti-inflammatory function in rheumatoid arthritis via the TLR4/MyD88/NF-κB signaling pathway; Zhou et al. (2017) suggested that sinomenine can inhibit osteoclast differentiation of human mesenchymal stem cells, indicating that sinomenine may has the potential for the treatment of over-activity of osteoclasts related bone diseases; Xie et al. (2016) reported that sinomenine can inhibit the metastasis, tumor-associated osteolysis and neovascularization of human osteosarcoma cells. However, to the best of our knowledge, it remains to be further investigated whether sinomenine can exert anti-osteoporosis function.

In the present study, we focused on the effects of sinomenine on the proliferation and differentiation of MC3T3-E1 cells *in vitro*, and the underlying mechanism will also be investigated. Our study may provide a theoretical basis for the application of sinomenine as an anti-osteoporosis medication.

2. Investigations and results

2.1. Sinomenine can affect the proliferation and apoptosis of MC3T3-E1 cells in vitro

First, to examine the effect of sinomenine on the proliferation and apoptosis of MC3T3-E1 cells *in vitro*, MC3T3-E1 cells were treated with different concentrations of sinomenine, and CCK-8 as well as flow cytometry assays have been performed. It was observed that low doses of sinomenine (100 μM) had no significant effect on proliferation (Fig. 1B) and apoptosis (Fig. 2, at 72 h) of MC3T3-E1 cells, while on the other hand, medium and high doses of sinomenine (500 and 1000 μM) has led to increased proliferation (Fig. 1B, 48 and 72 h) and marked decreased in the apoptosis of MC3T3-E1 cells *in vitro* (Fig. 2).

2.2. Sinomenine can affect osetogenesis of MC3T3-E1 cells in vitro

Next, we investigated the effect of sinomenine on the differentiation of MC3T3-E1 cells *in vitro*. As shown in Fig. 3, treatment of 500 and 1000 μM sinomenine significantly increased the activity of alkaline phosphatase (Fig. 3A) and secretion of osteocalcin in the cell culture supernatants of the MC3T3-E1 (Fig. 3B); furthermore, results of RT-qPCR and WB analysis indicated that 500 and 1000 μM sinomenine induced significant increase in the expression of osteogenic markers type I collagen and osteopontin on both mRNA and protein levels (Fig. 4). On the other hand, 100 μM sinomenine had no significant effect on the osetogenesis of MC3T3-E1 cells (Figs. 3 and 4).

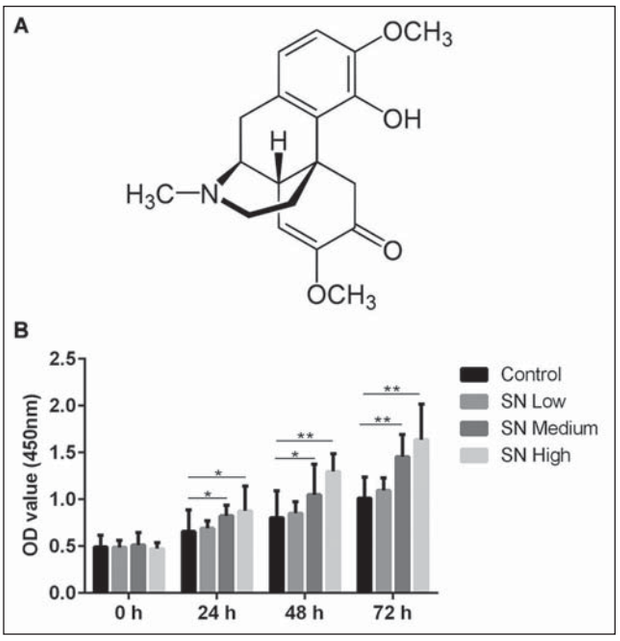

Fig. 1: A. Chemical formula of sinomenine. B. Sinomenine can affect the proliferation of MC3T3-E1 cells *in vitro* after 0 h, 24 h, 48 h and 72 h; Control, untreated cells; SN low, 100 μM sinomenine group; SN medium, 500 μM sinomenine group; SN high, 1000 μM sinomenine group. *, p<0.05; **, p<0.01.

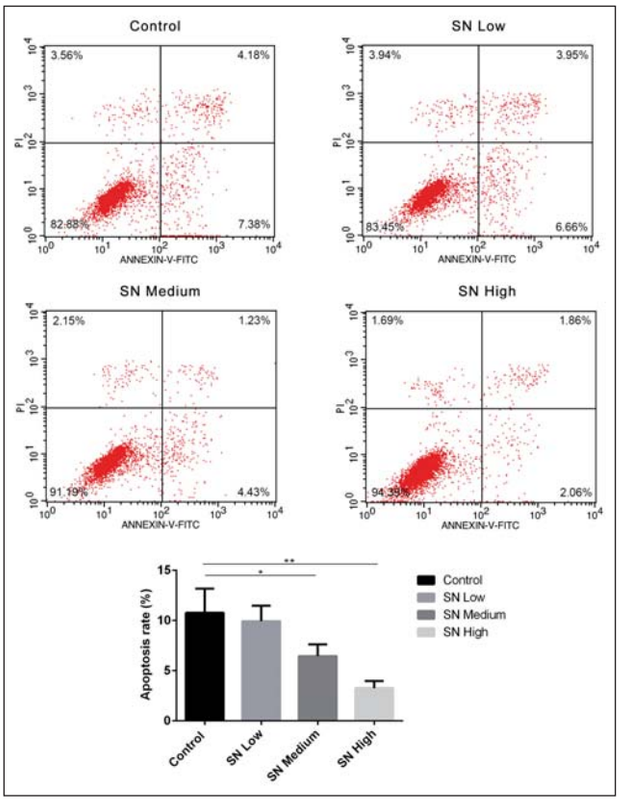

Fig. 2: Sinomenine can affect the apoptosis of MC3T3-E1 cells *in vitro*. Control, untreated cells; SN low, 100 μM sinomenine group; SN medium, 500 μM sinomenine group; SN high, 1000 μM sinomenine group. *p<0.05, **, p<0.01.

2.3. Sinomenine can activate Akt signaling of MC3T3-E1 cells via Akt/Runx2

Finally, we explored whether sinomenine can exert its anti-apoptotic and osteogenic effects via Akt/Runx2 signaling pathway. As shown in Fig. 5A, 500 and 1000 μM sinomenine markedly

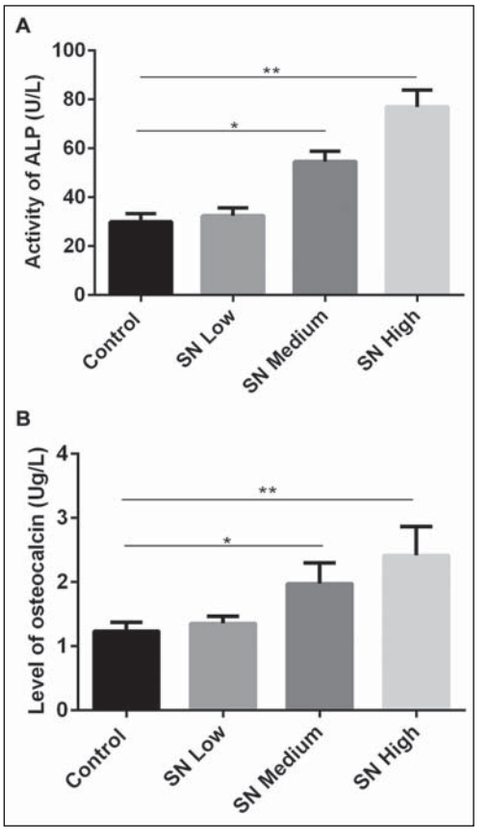

Fig. 3: Sinomenine can affect the secretion of osteocalcin and the activity of alkaline phosphatase in the cell culture supernatants of the MC3T3-E1 cells. A. Activity of alkaline phosphatase in different groups. B. Levels of osteocalcin in different groups. ALP, alkaline phosphatase; Control, untreated cells; SN low, 100 μM sinomenine group; SN medium, 500 μM sinomenine group; SN high, 1000 μM sinomenine group. *, p<0.05, **, p<0.01.

increased the expression of Runx2 and BCL-2 and decreased the expression of Bax in MC3T3-E1 cells on mRNA level, but had no effect on the mRNA expression of Akt; moreover, 500 and 1000 μM sinomenine also induced significant increase int he expression of Bcl-2, Runx2, and phosphorylated Akt, and dramatic decrease in the expression of Bax on protein level (Fig. 5B). On the other hand, 100 μM sinomenine had no significant effect on the expression of Akt/Runx2 signaling pathway in MC3T3-E1 cells (Fig. 5).

3. Discussion

In this study, we explored the roles of sinomenine in the proliferation and differentiation of MC3T3-E1 cells and the related mechanism. It was observed that sinomenine may affect the proliferation and differentiation of MC3T3-E1 cells *via* the Akt/Runx2 signaling pathway.

In current field of bone and cartilage related studies, the therapeutic effects of plant extracts have been discussed in many previous reports (Engel et al. 2011, 2016). MC3T3-E1 is a commonly used cellular model for the investigation of osteoporosis, and studies indicated that natural compounds may affect the proliferation and differentiation of MC3T3-E1 cells, so that natural compounds may serve as potential anti-osteoporosis medications. Huang et al. (2018) suggested that icariin can regulate the differentiation and proliferation of MC3T3-E1 cells *via* regulating the expression of microRNA-153; Caichompoo et al. (2009) observed that *Schisandra chinensis* Turcz. extracts affected the proliferation of osteoblasts *in vitro*; Shan et al. (2018) reported that puerarin can promote the proliferation and differentiation of MC3T3-E1 cells through regulating microRNA-106b; Chen et al. (2017) found that emodin can promote the osteogenesis of MC3T3-E1 cells through regulating BMP-9/Smad signaling pathway. To our knowledge, the effects of sinomenine on MC3T3-E1 cells remains unclear.

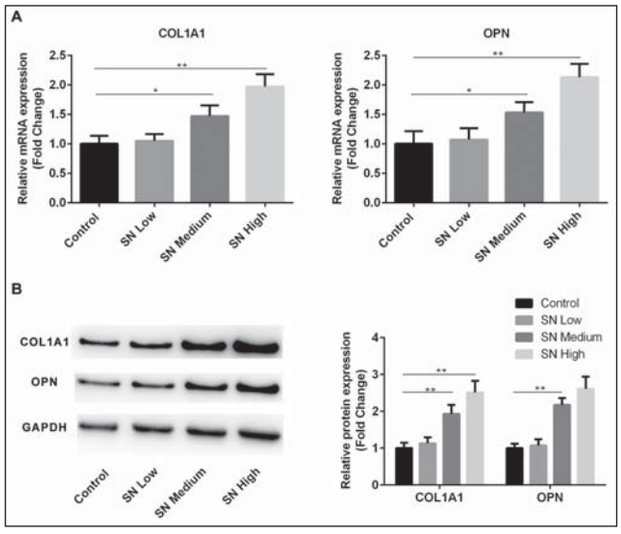

Fig. 4: Sinomenine can affect the expression of COL1A1 and OPN in MC3T3-E1 cells. (A) The mRNA expression of COL1A1 and OPN in different group; (B) The protein expression of COL1A1 and OPN in different groups. COL1A1, type I collagen; OPN, osteopontin; Control, untreated cells; SN low, 100 μM sinomenine group; SN medium, 500 μM sinomenine group; SN high, 1000 μM sinomenine group. *, p<0.05, **, p<0.01.

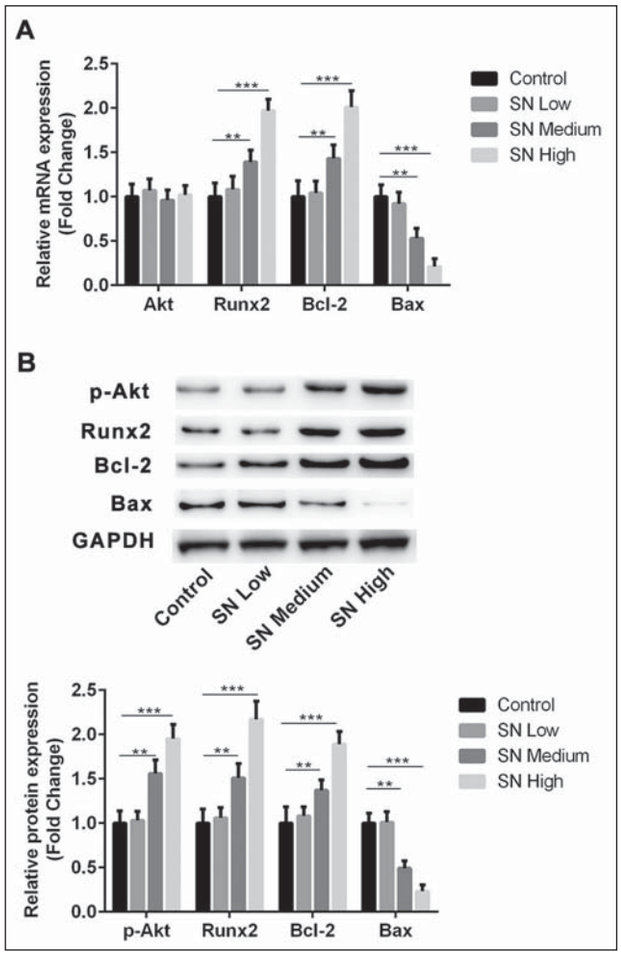

Fig. 5: A. Sinomenine can affect the mRNA expression of Runx2, Bax and Bcl-2 in MC3T3-E1cells. B. Sinomenine can affect the protein expression of p-Akt, Runx2, Bcl-2 and Bax in MC3T3-E1cells. Akt, protein kinase B; Runx2, runt-related transcription factor 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein Control, untreated cells; SN low, 100 μM sinomenine group; SN medium, 500 μM sinomenine group; SN high, 1000 μM sinomenine group. *, p<0.05, **, p<0.01, ***p<0.001.

In the present study, we observed that 500 and 1000 μM sinomenine promoted the proliferation and inhibited the apoptosis of MC3T3-E1 cells *in vitro* in a dose-dependent manner; moreover, sinomenine also increased the expression of alkaline phosphatase, osteocalcin, COL1A1 and OPN, which are all osteogenic markers. Taken together, the above results indicated that sinomenine may promote the proliferation and osteogenesis differentiation of MC3T3-E1 cells *in vitro*.

Runx2 is a transcription factor that belongs to the Runx transcriptional factor family, and it has been proved that Runx2 is one of the most important regulators involved in the process of proliferation and differentiation of osteoblasts (Chen et al. 2015; Wang et al. 2015). Previous studies indicated that Runx2 may be the earliest and most specific marker during the process of osteogenic differentiation (Hu et al. 2018; Wang et al. 2013). On the other hand, the Akt signaling pathway has been closely related to bone formation (Jafari et al. 2015), and interestingly, the interaction between Akt and Runx2 in osteogenesis has been reported previously. Choi et al. (2014) suggested that Akt may enhance the stability of Runx2 during the osteoblast differentiation. Ling et al. (2010) reported the roles of Akt-Runx2 signaling network in Wnt3a and heparin induced osteogenesis. In the present study, we observed that 500 and 1000 μM sinomenine markedly increased the expression of phosphorylated Akt, Runx2 and the Bcl-2(downstream anti-apoptotic protein) (Yang et al. 2017), and inhibited the expression levels of Bax(downstream pro-apoptotic protein) (Al-Rasheed et al. 2017) in MC3T3-E1 cells. The results suggested that sinomenine may regulate the proliferation and osteogenic differentiation of MC3T3-E1 cells *via* regulating Akt/Runx2 signaling.

To sum up, we provided novel evidence that sinomenine can promote the proliferation and osteogenic differentiation of MC3T3-E1 cells *via* regulating the Akt/Runx2 signaling pathway. Our results suggest the potential application of sinomenine for the prevention or treatment of osteoporosis.

4. Experimental

4.1. Cell culture

Mouse preosteoblastic cell line MC3T3-E1 cells (purchased from Keygen Biotech, Nanjing, China) were maintained in Dulbecco's modified eagle medium (DMEM, Gibco/Thermo Fisher Scientific, San Jose, USA) supplied with 10 % fetal bovine serum (FBS, Gibco/Thermo Fisher Scientific, San Jose, USA), 100 mg/mL streptomycin and 100 U/mL penicillin (Gibco/Thermo Fisher Scientific, San Jose, USA) in a humified incubator (37 °C and 5 % CO_2).

4.2. Sinomenine treatment

Sinomenine (Fig. 1A) was purchased from Selleckchem (no. S2359, Houston, USA) MC3T3-E1 cells were randomly divided into the control group, sinomenine low group (SN low, treated with 100 μM sinomenine), sinomenine medium group (SN medium, treated with 500 μM sinomenine) and sinomenine high group (SN high, treated with 1000 μM sinomenine). Cells were cultured for up to 72 h for further analysis.

4.3. Cell proliferation assay

After treatment of sinomenine, the proliferation of the cells was examined by CCK-8 methods using a Kit (Beyotime, Shanghai, China). Briefly, cells were seeded on to 96 well plates with the density of about 1 x 10^4 per well, and at 0, 24, 48, 72 h, 10 μl CCK-8 was added to each well, and then incubated for 1 h at 37 °C. The viability of the cells in each well was examined by measuring the optical density value at 450 nm using a microplate reader (Thermo Fisher Scientific, San Jose, USA).

4.4. Cell apoptosis assay

For the apoptosis analysis, MC3T3-E1 cells were double stained with Annexin V and propidium iodide using the cell apoptosis detection kit (BD Biosciences, Bedford, USA). The apoptosis of the cells with different treatment was determined by a BD FACS Verse flow cytometer (BD Biosciences, Bedford, USA).

4.5. Enzyme-linked immunosorbent assay

After treatment of sinomenine for 72 h, the levels of alkaline phosphatase and osteocalcin in the cell culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA) methods using commercialized kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

4.6. Real-time quantitative RT-PCR

The total RNAs were isolated from the MC3T3-E1 cells of different treatment by TRIzol (Thermo Fisher Scientific, San Jose, USA). Then the RNAs were than reverse transcribed into cDNA by PrimeScript™ RT Master Mix (Perfect Real Time, TaKaRa Bio Inc., Tokyo, Japan). Next, the mRNA expressions of type I collagen, Akt, Bcl-2, Bax and Runx2 was examined by PrimeScript™ RT reagent Kit (TaKaRa Bio Inc., Tokyo, Japan). The PCR reaction was performed on an ABI 7500 Real-Time PCR System (ABI, Waltham, USA) with the following thermo profile: 95 °C for 30 sec; 40 cycles of 95 °C for 5 s and 60 °C for 30 s. GAPDH was used for normalization.

4.7. Western blot

Total proteins were isolated from MC3T3-E1 cells with RIAP (Radio immunepreciptation assay) buffer (Beyotime, Haimen, China), and the concentration of the proteins were determined by Bradford protein assay kits (Beyotime, Haimen, China). Then the proteins were separated by SDS-PAGE with 10 % gel and transferred onto the PVDF membranes (Millipore, Billerica, USA). Next, the membranes were blocked with 5 % non-fat milk for 2 h and incubated with the primary antibodies at 4 °C overnight (all purchased from Abcam, Cambridge, USA). In the following day, the membranes were washed and incubated with the secondary antibodies (Beyotime, Haimen, China) at room temperature for 1 h, washed again and treated with the BeyoECL plus kit (Beyotime, Haimen, China), and analyzed by Tanon 5200 automatic chemical luminescence image system (Tanon, Shanghai, China). GAPDH was used for normalization.

4.8. Statistical analysis

Data were presented as the mean±standard deviation. Statistical analyses were performed by SPSS (version 20.0, IBM SPSS, Chicago, USA). The differences among the different groups were analyzed by analysis of variance (ANOVA). $P<0.05$ was considered as statistically significant.

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Conflict of interest: The authors declare no conflict of interest.

References

Al-Rasheed NM, Fadda LM, Attia HA, Ali HM, Al-Rasheed NM (2017) Quercetin inhibits sodium nitrite-induced inflammation and apoptosis in different rats organs by suppressing Bax, HIF1-alpha, TGF-beta, Smad-2, and AKT pathways. J Biochem Mol Toxicol 31(5). doi: 10.1002/jbt.21883

Caichompoo W, Zhang QY, Hou TT, Gao HJ, Qin LP, Zhou XJ (2009) Optimization of extraction and purification of active fractions from *Schisandra chinensis* (Turcz.) and its osteoblastic proliferation stimulating activity. Phytother Res 23: 289-292.

Chen DP, Wong CK, Leung PC, Fung KP, Lau CB, Lau CP, Lau CP, Li EK, Tam LS Lam CW (2011) Anti-inflammatory activities of Chinese herbal medicine sinomenine and Liang Miao San on tumor necrosis factor-alpha-activated human fibroblast-like synoviocytes in rheumatoid arthritis. J Ethnopharmacol 137: 457-468.

Chen H, Ji X, She F, Gao Y, Tang P (2017) miR-628-3p regulates osteoblast differentiation by targeting RUNX2: Possible role in atrophic non-union. Int J Mol Med 39, 279-286.

Chen X, Zhang S, Chen X, Hu Y, Wu J, Chen S, Chang J, Wang G, Gao Y (2017) Emodin promotes the osteogenesis of MC3T3-E1 cells via BMP-9/Smad pathway and exerts a preventive effect in ovariectomized rats. Acta Biochim Biophys Sin (Shanghai) 49: 867-878.

Choi YH, Kim YJ, Jeong HM, Jin YH, Yeo CY, Lee KY (2014) Akt enhances Runx2 protein stability by regulating Smurf2 function during osteoblast differentiation. FEBS J 281: 3656-3666.

Engel N, Ali I, Adamus A, Frank M, Dad A, Ali S, Nebe B, Atif M, Ismail M, Langer P, Ahmad VU (2016) Antitumor evaluation of two selected Pakistani plant extracts on human bone and breast cancer cell lines. BMC Complement Altern Med 16: 244.

Engel N, Oppermann C, Falodun A, Kragl U (2011) Proliferative effects of five traditional Nigerian medicinal plant extracts on human breast and bone cancer cell lines. J Ethnopharmacol 137: 1003-1010.

Hu L, Su P, Yin C, Zhang Y, Li R, Yan K, Chen Z, Li D, Zhang G, Wang L, Miao Z, Qian A, Xian CJ (2018) Microtubule actin crosslinking factor 1 promotes osteoblast differentiation by promoting beta-catenin/TCF1/Runx2 signaling axis. J Cell Physiol 233: 1574-1584.

Huang Z, Cheng C, Wang J, Liu X, Wei H, Han Y, Yang S, Wang X (2018) Icariin regulates the osteoblast differentiation and cell proliferation of MC3T3-E1 cells through microRNA-153 by targeting Runt-related transcription factor 2. Exp Ther Med 15: 5159-5166.

Jafari A, Siersbaek MS, Chen L, Qanie D, Zaher W, Abdallah BM, Kassem M (2015) Pharmacological inhibition of protein kinase G1 enhances bone formation by human skeletal stem cells through activation of RhoA-Akt signaling. Stem Cells 33: 2219-2231.

Li J, Yang S, Li X, Liu D, Wang Z, Guo J, Tan N, Gao Z, Zhao X, Zhang J, Gou F, Yokota H, Zhang P (2017) Role of endoplasmic reticulum stress in disuse osteoporosis. Bone 97: 2-14.

Ling L, Dombrowski C, Foong KM, Haupt LM, Stein GS, Nurcombe V, van Wijnen AJ, Cool SM (2010) Synergism between Wnt3a and heparin enhances osteogenesis via a phosphoinositide 3-kinase/Akt/RUNX2 pathway. J Biol Chem 285: 26233-26244.

Morita S, Jinno T, Nakamura H, Kumei Y, Shinomiya K, Yamamoto H (2005). Bone mineral density and walking ability of elderly patients with hip fracture: a strategy for prevention of hip fracture. Injury 36: 1075-1079.

Qin T, Yin S, Yang J, Zhang Q, Liu Y, Huang F, Cao W (2016) Sinomenine attenuates renal fibrosis through Nrf2-mediated inhibition of oxidative stress and TGFbeta signaling. Toxicol Appl Pharmacol 304: 1-8.

Shan Z, Cheng N, Huang R, Zhao B, Zhou Y (2018) Puerarin promotes the proliferation and differentiation of MC3T3-E1 cells via microRNA-106b by targeting receptor activator of nuclear factor-kappaB ligand. Exp Ther Med, 15: 55-60.

Song L, Liu D, Zhao Y, He J, Kang H, Dai Z, Wang X, Zhang S, Zan Y (2015) Sinomenine inhibits breast cancer cell invasion and migration by suppressing NF-kappaB activation mediated by IL-4/miR-324-5p/CUEDC2 axis. Biochem Biophys Res Commun 464: 705-710.

Strom O, Borgstrom F, Kanis JA, Compston J, Cooper C, McCloskey EV, Jonsson B (2011) Osteoporosis: burden, health care provision and opportunities in the EU: a report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos 6: 59-155.

Vandenbroucke A, Luyten FP, Flamaing J, Gielen E (2017) Pharmacological treatment of osteoporosis in the oldest old. Clin Interv Aging 12: 1065-1077

Wang CY, Yang SF, Wang Z, Tan JM, Xing SM, Chen DC, Xu SM, Yuan W (2013) PCAF acetylates Runx2 and promotes osteoblast differentiation. J Bone Miner Metab 31: 381-389.

Wang N, Liu R, Liu Y, Zhang R, He L (2016) Sinomenine potentiates P815 cell degranulation via upregulation of Ca^{2+} mobilization through the Lyn/PLCgamma/IP3R pathway. Int J Immunopathol Pharmacol 29: 676-683.

Wang Y, Chen S, Deng C, Li F, Wang Y, Hu X, Shi F, Dong N (2015) MicroRNA-204 targets Runx2 to attenuate BMP-2-induced osteoblast differentiation of human aortic valve interstitial cells. J Cardiovasc Pharmacol 66: 63-71.

Xie T, Ren HY, Lin HQ, Mao JP, Zhu T, Wang SD, Ye ZM (2016) Sinomenine prevents metastasis of human osteosarcoma cells via S phase arrest and suppression of tumor-related neovascularization and osteolysis through the CXCR4-STAT3 pathway. Int J Oncol 48: 2098-2112.

Yang M, Liu B, Jin L, Tao H, Yang Z (2017) Estrogen receptor beta exhibited anti-tumor effects on osteosarcoma cells by regulating integrin, IAP, NF-kB/BCL-2 and PI3K/Akt signal pathway. J Bone Oncol 9: 15-20.

Yao RB, Zhao ZM, Zhao LJ, Cai H (2017) Sinomenine inhibits the inflammatory responses of human fibroblast-like synoviocytes via the TLR4/MyD88/NF-kappaB signaling pathway in rheumatoid arthritis. Pharmazie 72: 355-360.

Yu SJ, Liu HC, Ling-Ling E, Wang DS, Zhu GX (2012) Proliferation and differentiation of osteoblasts from the mandible of osteoporotic rats. Exp Biol Med (Maywood) 237: 395-406.

Zhou B, Lu X, Tang Z, Liu D, Zhou Y, Zeng P, Xiong H (2017) Influence of sinomenine upon mesenchymal stem cells in osteoclastogenesis. Biomed Pharmacother 90: 835-841.