

CRNDE impacts the proliferation of osteoclast by estrogen deficiency in postmenopausal osteoporosis

W. LI¹, H.-M. ZHU^{2,3}, H.-D. XU^{4,1}, B. ZHANG¹, S.-M. HUANG¹

¹Department of Neuroscience, Institute of Chinese Medicine, Heilongjiang University of Chinese Medicine, Heilongjiang, Harbin, China

²Hubei University of Arts and Science, Hubei, XiangYang, China

³College of Veterinary Medicine, Northeast Agricultural University, Heilongjiang, Harbin, China

⁴Jiamusi College, Heilongjiang University of Chinese Medicine, Heilongjiang, Jiamusi, China

Abstract. – OBJECTIVE: Bone loss is the main reason for postmenopausal osteoporosis, caused by estrogen deficiency. ERT (estrogen replacement therapy) has been demonstrated to protect bone loss efficiently. LncRNA (long non-coding RNA) has been proved to be important in different disease progression. We aimed at analyzing whether the lncRNAs influence the activity of osteoclasts and the progression of this disease.

PATIENTS AND METHODS: RT-PCR (reverse transcriptase-polymerase chain reaction) was used to detect the expression of lncRNA CRNDE in OH (osteoclast from healthy people) and OP (osteoclast from patients with postmenopausal osteoporosis). MTT (methylthiazolyl tetrazolium) assay was used to detect the viability of the cells. The cell cycle and apoptosis rate in OH and OP were measured by the flow cytometry analysis. Western blot was used to analyze the possible related mechanism that CRNDE regulated the cells proliferation in postmenopausal osteoporosis.

RESULTS: We found that the CRNDE was highly expressed in the osteoclast from patients with OP, compared with OH. We also found that over-expressing CRNDE increased the viability in OH whilst reducing CRNDE in OP decreased the viability. The cell cycle was arrested in G0/G1 phase and the apoptosis rate was improved in OP after transfection with siRNA. Moreover, CRNDE could impact the proliferation of osteoclasts by PI3K/Akt signaling pathway and estrogen could inhibit this proliferation.

CONCLUSIONS: We found that lncRNA CRNDE was closely related to the postmenopausal osteoporosis with estrogen deficiency. CRNDE may be involved in the development and progression of postmenopausal osteoporosis in the absence of estrogen and become a potential target for treating.

Key Words:

CRNDE, Estrogen, Postmenopausal osteoporosis.

Introduction

Osteoblastic bone formation and osteoclastic bone resorption are a dynamic equilibrium for maintaining bone mass. The activation of osteoclastic bone resorption is frequently improved by estrogen loss in women after menopause, leading to osteoporosis^{1,2}. Estrogen improved the osteoblastic differentiation through ER- α or activating Wnt/ β -Catenin signaling and clinic data have demonstrated that postmenopausal women taking estrogen replacement therapy (ERT) had a reduced risk of pathological fracture^{3,4}. However, the essential underlying mechanism leading to osteoporosis is not fully understood and it is much important to find a new breakout focus for treatment. Long non-coding RNA (lncRNA) has been reported to participate in a lot of biological and pathological processes, such as carcinogenesis and chronic diseases. At present, accumulating evidence showed that lncRNA is involved in proliferation, apoptosis and inflammatory response in osteoporosis. LncRNA could regulate gene expression epigenetically in translation and transcription level, which is involved in multiple signaling pathways⁵⁻⁸. Ballantyne et al⁹ found that lncRNA SMILR could regulate the proliferation of vascular smooth muscle cells, which was highly expressed in the human unstable atherosclerotic plaques. This result suggested that modulation of SMILR might be a novel therapeutic strategy to prevent vascular lesion. Tong et al¹⁰ found that

DANCR overexpressed blood mononuclear cells have increased bone resorbing activity through secreting IL6 and TNF- α , finally resulting in osteoporosis. Their results demonstrated that DANCR is involved in the pathology of osteoporosis and may be a biomarker for postmenopausal osteoporosis. The lncRNA Colorectal Neoplasia Differentially Expressed (CRNDE) was first identified in colorectal adenomas and adenocarcinomas. The CRNDE locus spans ~10 kb and comprises five core exons with an additional exon that is infrequently present in transcripts¹¹. CRNDE was also found in other types of cancers including liver cancer, gastric cancer, gallbladder carcinoma, breast cancer, glioma¹²⁻¹⁶. However, the functional role of CRNDE in the postmenopausal osteoporosis is unclear. In this study, we first detected the expression of CRNDE in the osteoclasts from healthy people and osteoclasts from patients with postmenopausal osteoporosis. Then, the effect of CRNDE on the proliferation of two cell lines was analyzed. Finally, we investigated the likely mechanism of CRNDE in impacting the activity of osteoclasts.

Patients and Methods

Cell Culture and Treatment

The osteoclasts from healthy people and patients with postmenopausal osteoporosis were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and were used in our study. The cells were cultured at 37°C in a humidified incubator with 5% CO₂ and 95% air, and maintained in DMEM (Dulbecco's Modified Eagle Medium, HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (fetal bovine serum, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (100 U/mL/100 microg/mL) (Beyotime, Beijing, China).

RNA Extraction and Real-Time Quantitative PCR Assays

Total RNAs were isolated from osteoclasts by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA synthesis was done by using oligo (dT) primer and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix Ex Taq II reagent and the DICE Thermal cycler (TaKaRa, Otsu, Shiga, Japan), according to the manufacturer's instructions. Samples were matched to a standard curve generated by amplifying serially diluted products

using the same PCR reactions. β -actin (Actb) expression served as an internal control. Other primers were purchased from TaKaRa (Otsu, Shiga, Japan).

Cell Proliferation Assay

Target cells were seeded into 96-well plate with the density of 2500 cells per well. 10 ml Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) and 100 ml DMEM with 10% FBS were added to each well and cultured for 4 hours at 37°C. The absorbance at 450 nm was measured. Three controls were set in each group and the whole experiment was repeated three times.

Plasmid Transfection

The cells were seeded into 6-well plates at 60-80% confluency and placed in a fresh culture medium without fetal bovine serum (FBS) 2 hours before transfection. We transfected the plasmid into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 8 hours following the manufacturer's protocol. Diluted plasmid and Lipofectamine 2000 were mixed and incubated for 15-30 min. At last, the plasmid-lipid complex was added to the cells.

Cell Cycle and Apoptosis Analysis

The OH and OP cells were seeded into six-well plates with a concentration of 3×10^5 cells/well after being transfected with pcDNA-CRNDE or siRNA-CRNDE, respectively. Then, we collected the cells with low-speed centrifugation (1200 rpm, 6 min) at 4°C and cell pellets were re-suspended in 1 ml of PBS solution. Prior to FCM (flow cytometry) analysis, cells were lysed, centrifuged and re-suspended in PI (propidium iodide), Sigma-Aldrich (St. Louis, MO, USA) staining buffer containing 50 μ l/ml of PI and 250 μ l/ml of RNase A. Finally, the cell mixture was incubated at 4°C for 25 min in the darkness to investigate cell cycle and stained with 5 μ l of annexin V-FITC (fluorescein isothiocyanate) to detect apoptosis by FACS (fluorescence activated cell sorting) technique (Beckman Coulter, Brea, CA, USA). All the experiments were performed in three times.

Western Blot

The cells were grown in the 6-well plate. Total proteins were extracted by RIPA (radioimmuno-precipitation assay) lysis buffer containing phenylmethylsulfonyl fluoride (PMSF, Beyotime, Beijing, China). Supernatant protein levels were deter-

mined by standard BCA assay. An equal amount of protein (50 mg) was loaded into a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred onto a PVDF membrane (polyvinylidene difluoride, Millipore, Billerica, MA, USA) after electrophoresis. After blocking non-specific binding sites for 1 h at room temperature with 5% non-fat milk in TBST (Tris buffered saline and Tween-20, Boster, Wuhan, China), the membranes were individually incubated for overnight with primary antibody at 4°C. After that, the respective secondary antibody was used to incubate these membranes for 1 h at room temperature and the results emerged.

Statistical Analysis

All experiments were repeatedly performed in three times. The data are expressed as the mean $\pm$ SD, and samples were evaluated by the ANOVA test using the software GraphPad 7 (GraphPad, La Jolla, CA, USA). p -value < 0.05 was considered as statistically significant. Multiple comparison between the groups was performed using Tukey's post-hoc test.

Results

CRNDE Was Highly Expressed in the OAS and Increased the Proliferation of Cells

To explore the role of lncRNA CRNDE in postmenopausal osteoporosis, we detected the expression of CRNDE in OH and OP by qRT-PCR. The results showed that the expression of CRNDE was highly expressed in OP, compared

with the OH. Moreover, we investigated the proliferation of cell lines and we found that OP had a much greater proliferation rate compared to OH (Figure 1). These data demonstrated that improved expression of CRNDE may be responsible for the progression of postmenopausal osteoporosis. However, the detailed mechanism is still unclear.

Convert the Expression of CRNDE Impacts the Proliferation of OH and OP

To explore whether CRNDE could impact on the proliferation of osteoclast, CRNDE was overexpressed in OH and CRNDE expression was knocked-down in OP. Then, the observed data were analyzed. The results showed that overexpressed CRNDE in OH improved the proliferation of cells and knockdown of the expression of CRNDE suppressed the proliferation in OP (Figure 2). This suggested that altering the expression of CRNDE might affect the proliferation in osteoclasts.

The Cell Cycle Was Arrested and the Apoptosis Rate Was Increased in OP After the Expression of CRNDE Was Knockdown

The situation of cell cycle and apoptosis were measured in OH and OP after the expression of CRNDE was converted. Flow cytometry analysis was utilized to research the possible proliferation mechanism of CRNDE in osteoclasts. These data suggested that the cell percentage was significantly decreased in G0/G1 phase, cell percentage was significantly increased in S-phase, and reduced cell apoptosis in OH after CRNDE was

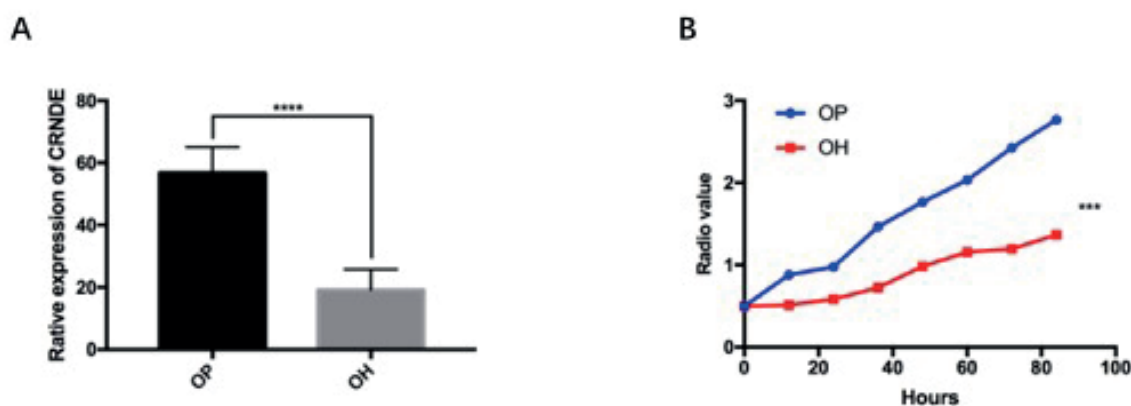


Figure 1. CRNDE was highly expressed in the OP and promoted the proliferation of cells. **A**, The expression of CRNDE in the OH and OP was detected by qRT-PCR assay. **** $p < 0.0001$. **B**, The proliferation of OH and OP was detected by CCK-8 assays. *** $p < 0.001$.

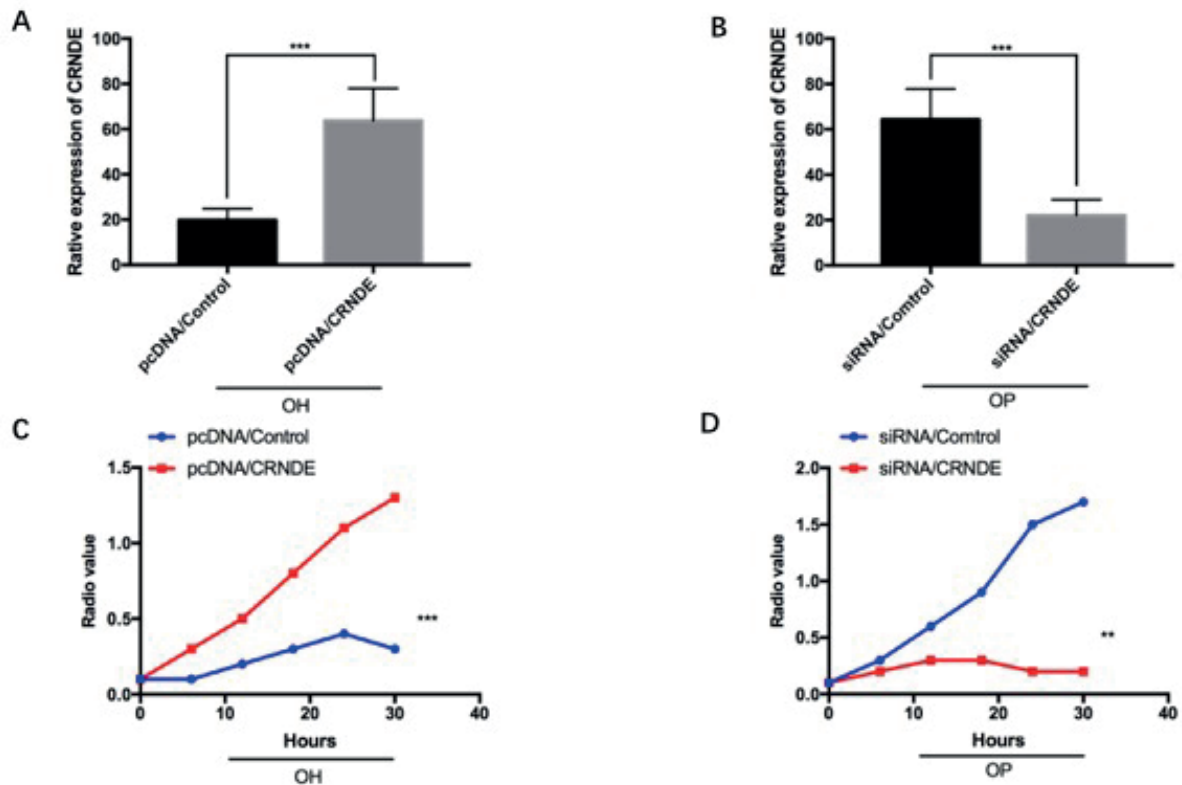


Figure 2. Converted expression of CRNDE impacts the proliferation of OH and OP. *A-B*, Relative expression of CRNDE was detected by PCR, *** $p < 0.001$. *C-D*, The proliferation of OH and OP was detected by CCK-8 assays. *** $p < 0.001$, ** $p < 0.01$.

overexpressed. The cell percentage was significantly increased in G0/G1 phase and significantly decreased in S-phase, promoting cell apoptosis in OP after CRNDE was knocked down (Figure 3). The results demonstrated that CRNDE regulated the cell proliferation in osteoclast by altering cell cycle in G0/G1 phase to induce cell apoptosis in osteoclasts.

CRNDE Regulated Cell Proliferation Depends on Estrogen in Osteoclast via PI3K/Akt Signaling Pathway

To research the mechanism of CRNDE, cell cycle in G0/G1 phase was arrested to induce cell apoptosis in osteoclasts after being knocked-down. The related protein expression levels of p-PI3K, p-Akt, Bcl-2 and P53 in OH and OP

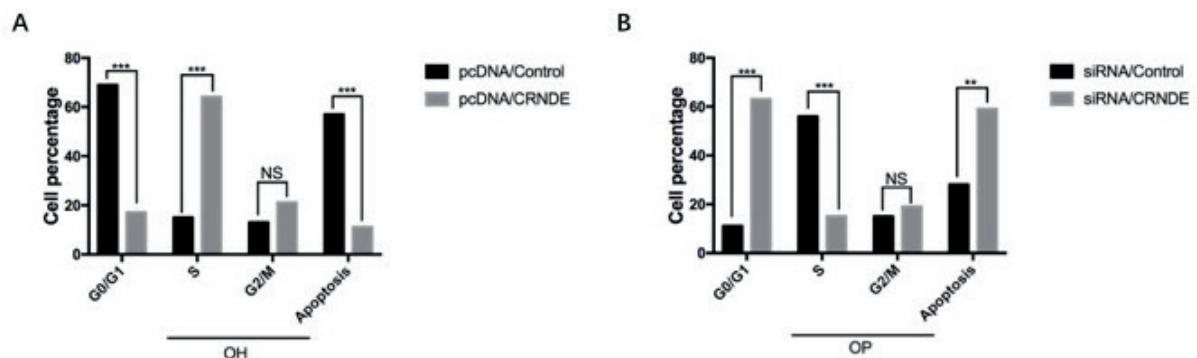


Figure 3. Cells apoptosis influences the cells proliferation. *A-B*, The cell cycle was detected by flow cytometry assays in OH and OP. *** $p < 0.001$, ** $p < 0.01$.

cells, after transfection with pcDNA CRNDE or lncRNA-CRNDE, respectively, were detected by Western blot. Mechanistically, the related protein expression levels of p-PI3K, p-Akt and Bcl-2 were significantly decreased and the expression of P53 was significantly increased in OH cells after transfection with pcDNA-CRNDE. The relative protein expression levels of p-PI3K, p-Akt and Bcl-2 were significantly increased and the expression of P53 was significantly decreased in OP cells after transfection with siRNA-CRNDE. These data suggested that regulation of the expression of CRNDE impacts the proliferation in osteoclast via PI3K/Akt signaling pathway. Furthermore, estrogen was added into the OH, in which CRNDE was overexpressed (Figure 4). We found that the proliferation ability was decreased in OH – in which CRNDE was overexpressed – showing that the proliferation of osteoclast depended on the estrogen.

Discussion

It has been showed that the balance of osteoblasts and osteoclasts is an important factor for osteoporosis. The osteoclast mainly executed the bone resorption, leading to bone loss¹⁷. Thus, how to modulate the number of osteoclasts has become

a new focus for treatment. In this study, we firstly demonstrated that lncRNA CRNDE had a closed relationship with the proliferation of osteoclasts in postmenopausal osteoporosis. Accumulating evidence has indicated that lncRNAs have closely related in regulating different processes of diseases, including cell growth, migration, invasion angiogenesis and tumorigenesis. Sun et al¹⁸ found that lncRNA CRNDE promotes cell proliferation, invasion and migration by competitively binding miR-384 in papillary thyroid cancer. In the thyroid cancer, CRNDE was found to be up-regulated in the papillary thyroid cancer tissues and CRNDE was associated with poor prognosis in papillary thyroid cancer patients¹⁹. About other tumors, CRNDE acted as a scaffold of DMBT1 and C-IAP1 complexes to activate PI3K-AKT pathway, which subsequently promoted gallbladder carcinoma carcinogenesis¹⁴. Our research revealed that CRNDE was significantly increased in osteoclast from patients with postmenopausal osteoporosis compared with the osteoclasts from healthy people; the proliferation rate was higher in OP, compared with OH by CCK-8 assay. The results showed that CRNDE was closely correlated with osteoclasts. Then, we overexpressed CRNDE in OH improving the cell proliferation rate and knocked down CRNDE in OP, which in-

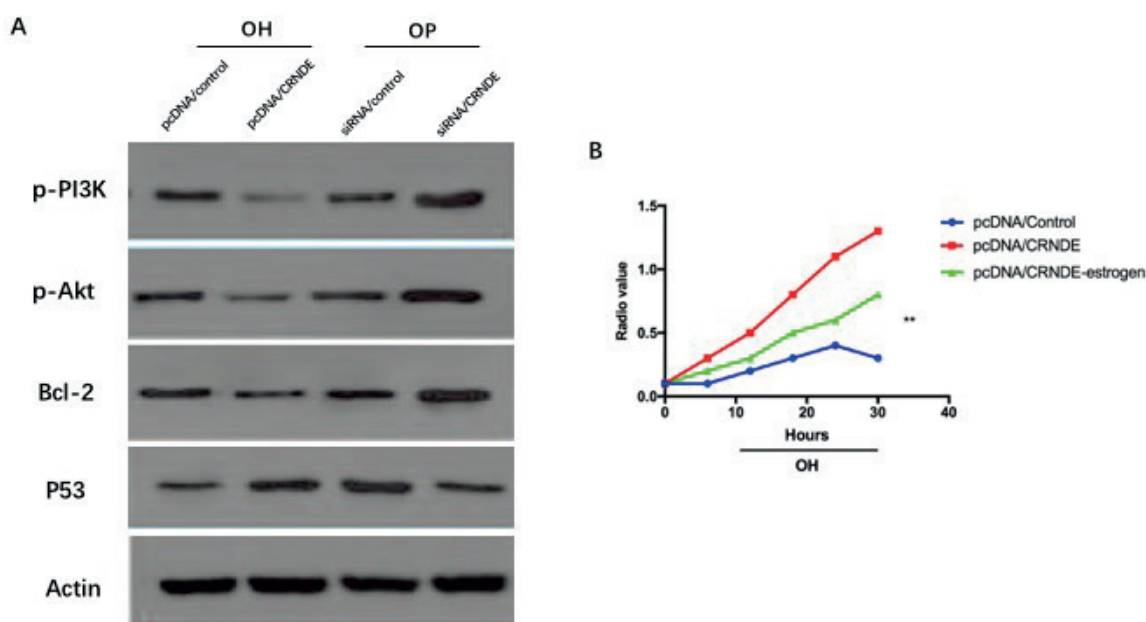


Figure 4. CRNDE regulated cell proliferation of osteoclast by PI3K/Akt signaling pathway. **A**, Relative expression of p-PI3K, p-Akt, Bcl-2 and P53 was detected by Western blot. *** $p < 0.001$. **B**, The proliferation of osteoclast was detected by CCK-8 assays. ** $p < 0.01$.

hibited the rate of cell proliferation. This result indicated that lncRNA CRNDE was closely related to the cell proliferation. Osteogenesis is made of two parts including bone formation and bone resorption. This process is similar to cell growth containing cell proliferation and cell apoptosis. The growth of osteoclasts influences the effect of bone resorption, and apoptosis becomes the crucial point for regulating the activity of bone²⁰. Thus, we detected the apoptosis and found that cell percentage in G0/G1 phase was significantly decreased and cell percentage in S-phase was significantly increased, reducing cell apoptosis in OH after that CRNDE was overexpressed. The cell percentage in G0/G1 phase was significantly improved and cell percentage in S-phase was significantly declined and promoted cell apoptosis in OP after CRNDE was knockdown. These results suggested that CRNDE could impact the proliferation of osteoclasts via regulating cell apoptosis. The PI3K/Akt signal pathway is activated by oncogenes, and activated PI3K promoted cancer cell growth and invasion²¹. We aimed at investigating whether CRNDE influenced the activities of osteoclasts by PI3K/Akt signal pathway. We detected the expression of p-PI3K, p-Akt and Bcl-2 was reduced and the expression of P53 was significantly increased after overexpressed CRNDE in OH. The expression of p-PI3K, p-Akt and Bcl-2 was significantly increased and the expression of P53 was reduced after knocked-down CRNDE in OP. Furthermore, the proliferation of OH was found to be reduced after that estrogen was added into it; thus, the expression of CRNDE was overexpressed. These results suggested that CRNDE could influence the proliferation of osteoclasts in the absence of estrogen by PI3K/Akt signaling pathway. In summary, lncRNA CRNDE is a potential and independent influential factor for the progress of osteoclasts and involves in the cell proliferation via PI3K/Akt signaling pathway. This research provides us with a novel treatment strategy for postmenopausal osteoporosis.

Conclusions

We observed that lncRNA CRNDE was highly expressed in OP, and closely related with the proliferation of osteoclasts. After that, we found that lncRNA CRNDE may regulate cell apoptosis to promote proliferation of CRNDE via PI3K/Akt signaling pathway and that estrogen could inhibit this proliferation of osteoclasts. These results

indicated that lncRNA CRNDE may be a potential target for postmenopausal osteoporosis treatments in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

- 1) NELSON ER, WARDELL SE, McDONNELL DP. The molecular mechanisms underlying the pharmacological actions of estrogens, SERMs and oxysterols: implications for the treatment and prevention of osteoporosis. *Bone* 2013; 53: 42.
- 2) MIYAUCHI Y, SATO Y, KOBAYASHI T, YOSHIDA S, MORI T, KANAGAWA H, KATSUYAMA E, FUJIE A, HAO W, MIYAMOTO K, TANDO T, MORIOKA H, MATSUMOTO M, CHAMBON P, JOHNSON RS, KATO S, TOYAMA Y, MIYAMOTO T. HIF1 α is required for osteoclast activation by estrogen deficiency in postmenopausal osteoporosis. *Proc Natl Acad Sci U S A* 2013; 110: 16568-16573.
- 3) HONG L, COLPAN A, PEPTAN IA. Modulations of 17-beta estradiol on osteogenic and adipogenic differentiations of human mesenchymal stem cells. *Tissue Eng* 2006; 12: 2747-2753.
- 4) BHUKHAI K, SUKSEN K, BHUMMAPHAN N, JANJORN K, THONGON N, TANTIKANLAYAPORN D, PIYACHATURAWAT P, SUKSAMRARN A, CHAIROUNGDUA A. A phytoestrogen diarylheptanoid mediates estrogen receptor/akt/glycogen synthase kinase 3 β protein-dependent activation of the wnt/ β -catenin signaling pathway. *J Biol Chem* 2012; 287: 36168-36178.
- 5) DELAS MJ, HANNON GJ. LncRNAs in development and disease: from functions to mechanisms. *Open Biol* 2017; 7.pii: 170121.
- 6) SALEHI S, TAHERI MN, AZARPIRA N, ZARE A, BEHZAD-BEHBHANI A. State of the art technologies to explore long non-coding RNAs in cancer. *J Cell Mol Med* 2017; 21: 3120-3140.
- 7) D'ANIELLO C, PISCONTI S, FACCHINI S, IMBIMBO C, CAVALIERE C. Long noncoding RNA GIHCG is a potential diagnostic and prognostic biomarker and therapeutic target for renal cell carcinoma. *Eur Rev Med Pharmacol Sci* 2018; 22: 1169-1170.
- 8) GAO P, WEI GH. Genomic insight into the role of lncRNA in cancer susceptibility. *Int J Mol Sci* 2017; 18. pii. E1239.
- 9) BALLANTYNE MD, PINEL K, DAKIN R, VESEY AT, DIVER L, MACKENZIE R, GARCIA R, WELSH P, SATTAR N, HAMILTON G, JOSHI N, DWECK MR, MIANO JM, MCBRIDE MW, NEWBY DE, McDONALD RA, BAKER AH. Smooth muscle enriched long noncoding rna (SMILR) regulates cell proliferation. *Circulation* 2016; 133: 2050-2065.
- 10) TONG X, GU PC, XU SZ, LIN XJ. Long non-coding RNA-DANCR in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. *Biosci Biotechnol Biochem* 2015; 79: 732-737.

- 11) GRAHAM LD, PEDERSEN SK, BROWN GS, HO T, KASSIR Z, MOYNIHAN AT, VIZGOF EK, DUNNE R, PIMLOTT L, YOUNG GP, LAPOINTE LC, MOLLOY PL. Colorectal neoplasia differentially expressed (CRNDE), a novel gene with elevated expression in colorectal adenomas and adenocarcinomas. *Genes Cancer* 2011; 2: 829-840.
- 12) CHEN Z, YU C, ZHAN L, PAN Y, CHEN L, SUN C. LncRNA CRNDE promotes hepatic carcinoma cell proliferation, migration and invasion by suppressing miR-384. *Am J Cancer Res* 2016; 6: 2299-2309.
- 13) HU CE, DU PZ, ZHANG HD, HUANG GJ. Long noncoding RNA CRNDE promotes proliferation of gastric cancer cells by targeting miR-145. *Cell Physiol Biochem* 2017; 42: 13-21.
- 14) SHEN S, LIU H, WANG Y, WANG J, NI X, AI Z, PAN H, LIU H, SHAO Y. Long non-coding RNA CRNDE promotes gallbladder carcinoma carcinogenesis and as a scaffold of DMBT1 and c-IAP1 complexes to activating PI3K-AKT pathway. *Oncotarget* 2016; 7: 72833-72844.
- 15) HUAN J, XING L, LIN Q, XUI H, QIN X. Long noncoding RNA CRNDE activates wnt/beta-catenin signaling pathway through acting as a molecular sponge of microRNA-136 in human breast cancer. *Am J Transl Res* 2017; 9: 1977-1989.
- 16) KIANG KM, ZHANG XQ, ZHANG GP, LI N, CHENG SY, POON MW, PU JK, LUI WM, LEUNG GK. CRNDE expression positively correlates with EGFR activation and modulates glioma cell growth. *Target Oncol* 2017; 12: 353-363.
- 17) WANG Z, GOH J, DAS DE S, GE Z, OUYANG H, CHONG JS, LOW SL, LEE EH. Efficacy of bone marrow-derived stem cells in strengthening osteoporotic bone in a rabbit model. *Tissue Eng* 2006; 12: 1753-1761.
- 18) SUN H, HE L, MA L, LU T, WEI J, XIE K, WANG X. LncRNA CRNDE promotes cell proliferation, invasion and migration by competitively binding miR-384 in papillary thyroid cancer. *Oncotarget* 2017; 8: 110552-110565.
- 19) LUO YH, LIANG L, HE RQ, WEN DY, DENG GF, YANG H, HE Y, MA W, CAI XY, CHEN JQ, CHEN G. RNA-sequencing investigation identifies an effective risk score generated by three novel lncRNAs for the survival of papillary thyroid cancer patients. *Oncotarget* 2017; 8: 74139-74158.
- 20) DALLE CARBONARE L, VALENTI MT, ZANATTA M, DONATELLI L, LO CASCIO V. Circulating mesenchymal stem cells with abnormal osteogenic differentiation in patients with osteoporosis. *Arthritis Rheum* 2009; 60: 3356-3365.
- 21) YANG L, LIU Y, WANG M, QIAN Y, DAI X, ZHU Y, CHEN J, GUO S, HISAMITSU T. *Celastrus orbiculatus* extract triggers apoptosis and autophagy via PI3K/Akt/mTOR inhibition in human colorectal cancer cells. *Oncol Lett* 2016; 12: 3771-3778.