

## Full Length Article

## Bone and plasma citrate is reduced in osteoporosis

Hongdong Chen<sup>a</sup>, Yeyang Wang<sup>b</sup>, Huaiqian Dai<sup>a</sup>, Xinggui Tian<sup>c</sup>, Zhong-Kai Cui<sup>a,\*</sup>,  
Zhenguo Chen<sup>a</sup>, Le Hu<sup>a</sup>, Qiancheng Song<sup>a</sup>, Anling Liu<sup>d</sup>, Zhiyong Zhang<sup>e</sup>, Guozhi Xiao<sup>f</sup>,  
Jian Yang<sup>a,g</sup>, Yu Jiang<sup>h</sup>, Xiaochun Bai<sup>a,\*</sup>

<sup>a</sup> Department of Cell Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, China

<sup>b</sup> Department of Spine Surgery, Guangdong Second Provincial General Hospital, Guangzhou, Guangdong, China

<sup>c</sup> Department of Spine Surgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China

<sup>d</sup> Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, China

<sup>e</sup> Translational Research Centre of Regenerative Medicine and 3D Printing Technologies of Guangzhou Medical University, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China

<sup>f</sup> Department of Biology and Shenzhen Key Laboratory of Cell Microenvironment, Southern University of Science and Technology, Shenzhen, Guangdong, China

<sup>g</sup> Department of Biomedical Engineering, Materials Research Institutes, The Huck Institutes of The Life Sciences, The Pennsylvania State University, University Park, PA, USA

<sup>h</sup> Department of Pharmacology and Chemical Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA



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## ABSTRACT

High concentration of citrate exists in bone of humans and all osteo-vertebrates, and citrate incorporation imparts important biomechanical and other functional properties to bone. However, which cells are responsible for citrate production in bone remains unclear and whether the citrate component changes with bone loss during osteoporosis is also not known. Here, we show that the citrate content is markedly reduced in the bone of mice or rats with age-related, ovariectomy-induced or retinoic acid-induced bone loss. Plasmic citrate is also down-regulated in osteoporotic animals. Importantly, the plasmic citrate level of aged osteoporotic males is significantly lower than that of young healthy males and positively correlates with human lumbar spine bone mineral density (BMD) and total hip BMD. Furthermore, citrate production increases with *in vitro* osteoblastic differentiation, accompanied by upregulation of proteins involved in citrate secretion, suggesting that osteoblasts are highly specialized cells that produce citrate in bone. Our findings establish a novel relationship between citrate content and bone loss-related diseases such as osteoporosis, suggesting a critical role of bone citrate in the maintenance of the citrate balance in the circulation. Serum citrate level may thus represent a novel marker for osteoporosis.

## 1. Introduction

In 1941, Dickens *et al.* first reported that bone contained extremely high levels of citrate [1]. Numerous subsequent studies have confirmed that high concentrations of citrate exist in bone and teeth of humans and all osteo-vertebrates [2–7]. As a major and indispensable component of bone (~20–80 μmol/g), citrate comprises ~1.6% of the bone content and ~5% of the organic component of bone. Moreover, approximately 80% of total body citrate resides in bone. In comparison with most soft tissues (except the prostate), citrate concentration in bone is approximately 50-fold higher, suggesting an important role of citrate in the structural/functional properties of bone.

Citrate has always occupied an important position in metabolism [8–11]. Recently, the development of advanced and necessary research

methodologies and technologies have added new dimensions to its role in bone tissue. As illustrated by Hu *et al.* and by Davies in PNAS [12, 13], citrate is strongly bound onto the surfaces of the apatite nanocrystals in bone. This bound citrate accounts for 5.5 wt% of the organic components in bone and provides much more carboxylate groups for binding to the calcium of apatite than all non-collagenous proteins do. By interfering with nanocrystal thickening and stabilizing apatite nanocrystal sizes in bone, citrate incorporation imparts important biomechanical and other functional properties to bone, influencing its stability, strength and resistance to fracture [14, 15]. However, there is limited *in vivo* evidence and functional studies related to the origin of citrate in bone and its role in the development, maintenance and repair of bone.

In our previous study, citrate-based biodegradable materials were

\* Corresponding authors at: Department of Cell Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong 510515, China.  
E-mail addresses: [zhongkaicui@smu.edu.cn](mailto:zhongkaicui@smu.edu.cn) (Z.-K. Cui), [baixc15@smu.edu.cn](mailto:baixc15@smu.edu.cn) (X. Bai).

synthesized and used for bone repair. These citrate-based biomaterials exhibited excellent biocompatibility and extensive osseointegration and osteoconductivity with host bone tissue. They also promoted better replication of the native bone citrate and inorganic mineral content [16–19].

Using different animal models, we demonstrate that the citrate content is reduced in both bone and serum of animal models with osteoporosis or osteopenia. The plasmic citrate level of aged males with osteoporotic history is also significantly lower than that of young healthy males and is positively correlated with human lumbar spine bone mineral density (BMD) and total hip BMD. Osteoblasts are highly-specialized cells that produce citrate. Our findings suggest that decreased citrate content in osteoporotic bone reflects reduced citrate incorporation into the structure of bone, which may compromise bone strength and contribute to fragility fractures in the osteoporotic population. Besides incorporating and limiting the size of apatite nanocrystals, bone citrate may also play a critical role in the maintenance of the citrate balance in the circulation.

## 2. Materials and methods

### 2.1. Human subjects

This study was approved by institutional review board of Southern Medical University and Orthopedic Hospital of Puning. In accordance with local ethical standards, informed consent was obtained from patients before the recruitment in this study. For analysis of serum citrate content, we recruited 120 healthy young male volunteers and 120 elderly male volunteers from the Department of Orthopedics, Traditional Chinese Medicine Hospital of Puning, Orthopedic Hospital of Puning (Puning, Guangdong, 515300, China). The 120 young male volunteers all passed the physical examination for enlistment. The 120 elderly male volunteers underwent annual physical examination in the physical examination center of Orthopedic Hospital of Puning, and they had all exhibited different osteoporotic fractures over the past 2 years. By the time of blood collecting, all fractures had recovered. The information of fracture sites and the age at which the fractures occurred was collected. In this study, osteoporotic fractures were defined as low trauma fractures (defined as caused by a fall from one's own height or lower over 50 years of age). All pathologic (due to cancer, bone tuberculosis, etc.) or traumatic fractures (resulted from a fall greater than a standing height, or accident including motor vehicle accidents, sport accidents, etc.) were excluded [20]. Participants with osteoarthritis, cancer, kidney disease or prostate disease were also excluded. Another 87 volunteers (male = 25, female = 62) who had been enrolled in a study on the determinants of bone loss were included. For those 87 volunteers, BMD of the lumbar spine (L1–L4) and total hip was measured by dual-energy X-ray absorptiometry (DXA) on a QDR 2000 device (Hologic, Bedford, MA, U.S.A.). The results of DXA measurements were expressed as BMD (g/cm) and T-score and Z-score of a healthy reference population, as supplied by the manufacturer. Age, body weight and height were recorded by the same physician.

For all those subjects, fasting blood samples (5–10 mL) were collected between 7:00 am and 9:00 am. Serum separation was completed within 2 h by centrifugation (5000 rpm for 10 min) and serum samples were stored at  $-80^{\circ}\text{C}$  until tests content analysis or ELISA assay were run. Sample collections and separations were performed according to standard laboratory procedures.

### 2.2. ELISA analysis

Human serum osteocalcin (OCN), procollagen I N-Terminal Peptide (PINP) and cross linked C-telopeptide of type I collagen ( $\beta$ -CTX) were measured using a human OC/BGP ELISA Kit (E-EL-H1343c; Elabscience Biotechnology, Biotechnology, Bethesda, MD, USA), a human PINP ELISA Kit (E-EL-H0185c; Elabscience Biotechnology) and a human  $\beta$ -

CTX kit (E-EL-H0960; Elabscience Biotechnology) according to the manufacturers' instructions.

### 2.3. Animals

The Southern Medical University Animal Care and Use Committee approved all procedures involving animals. The housing and breeding of mice were conducted according to the guidelines for the ethical treatment of animals. All the C57/BL6 mice and Sprague-Dawley (SD) rats were given tap water and fed *ad libitum*, with a cycle of 12 h light and 12 h darkness. Different animal models of osteoporosis were constructed.

Twenty 3-month-old C57/BL6 female mice were divided into two groups (with an equal weight distribution): control group (Sham,  $n = 10$ ) and ovariectomy group (OVX,  $n = 10$ ). OVX group mice were ovariectomized bilaterally and Sham group mice had their ovaries exposed but not removed. All the mice were maintained for 3 months. Estrogen-depleted bone loss caused by decreased skeletal growth and increased skeletal resorption was observed after three months post-OVX. Successful ovariectomy was confirmed at necropsy by determining atrophy of the uterine horns.

Twenty adult male SD rats (3.5 months old; weighing between 200 and 250 g) were weight-matched and housed in our animal facility. These rats were divided into two groups: control group (CON,  $n = 10$ ) and retinoic acid group (RA,  $n = 10$ ). All rats were treated by oral gavage for 21 days. The control group received 0.5% sodium carboxymethyl cellulose (70 mg/kg), while the retinoic acid group was given retinoic acid at 70 mg/kg/d. The dosing was adjusted according to the daily weight conditions [21].

As a model of senile osteoporosis, ten C57/BL6 male mice were housed in our animal facility until they reached 20 months of age (aged group). Adult mice (6 months,  $n = 10$ ) were used as the control group.

Body weights were checked weekly throughout the entire experimental period. All animals were sacrificed under general anesthesia with pentobarbital sodium and blood samples were collected by cardiac puncture for serum isolation. Serum was separated by centrifugation at  $3000 \times g$  for 10 min and stored at  $-80^{\circ}\text{C}$  for further analysis. Right and left femora and tibiae and crania were harvested and cleaned of all non-osseous tissue for further analysis.

### 2.4. Microcomputed tomography (micro-CT) analysis

BMD measurement was performed in the femora of narcotized mice on a micro-CT Scanner (Viva CT40; Scanco Medical AG, Bassersdorf, Switzerland) as previously described [22]. Three regions were evaluated: cortical bone in the mid-diaphysis, secondary spongiosa in the distal metaphysis and the entire femur (Supplementary Fig. S1D). We started morphometric analysis at the first slice in which the femoral condyles were fully merged and extended for 100 slices proximally. The three-dimensional structure and morphometry were constructed and analyzed to obtain bone volume/tissue volume (BV/TV, %), BMD (mg HA/cm), trabecular number (Tb.N.,  $\text{mm}^{-1}$ ), trabecular thickness (Tb.Th, mm) and trabecular separation (Tb.Sp, mm). We also performed micro-CT imaging in the mid-diaphysis of the femur and performed mid-shaft evaluation of 100 slices to quantify the cortical thickness, bone mineral density and outer/inner perimeter of the mid-shaft.

### 2.5. Animal serum biochemical analysis

Serum biochemical analysis was carried out using a Catalyst Dx<sup>®</sup> Chemistry Analyzer. Serum levels of alkaline phosphatase (ALKP), phosphate (Phos), calcium ions (Ca), sodium (Na), potassium ions (K) and chloride ions (Cl) were determined. The methodology and reagents used were recommended by the manufacture of that system.

## 2.6. Animal bone preparation

Femora and tibiae dissected from the mice or rats were defleshed and rinsed thoroughly with PBS. Almost all of the cells, including bone marrow and cells on the trabecular surface were washed away. So, the intracellular citric acid in bone tissue is negligible. The bones were then defatted by soaking in a 1:1 v/v mixture of chloroform and ethanol for about 1 h as described previously [3, 23]. Next, the cleaned, defatted bone samples were powdered in a liquid nitrogen-cooled grinding device and reduced to particles < 10 nm in diameter. A 50 mg aliquot of the powder was placed in a 10 mL centrifuge tube to which was added 2 mL of 1.0 M HCl, and the tube was set in a water bath held at 60 °C for 1 h. The above procedure dissolves the hydroxyapatite component of the bone and liberates bound citrate into the solvent phase. The samples were then brought to pH 5 by drop wise addition of 1 M KOH. After centrifuging at 1200 × g for 5 min, the clear supernatant was decanted into a fresh tube and stored at 4 °C until analysis of citrate content.

## 2.7. Determination of citrate content

Serum samples were deproteinized using a deproteinizing sample preparation kit (K808-100; Biovision, Zurich, Switzerland) according to the manufacturer's instructions before citrate content determination. Using this kit, serum proteins which might cause erratic readings were precipitated by perchloric acid (PCA), then excess PCA was removed and samples were neutralized.

Citrate content was determined using a citrate colorimetric assay kit (K655-100; Biovision) according to the manufacturer's instructions, which converts the pyruvate from an almost colorless probe to an intensely colored product to allow quantification.

## 2.8. Histological examination, tartrate-resistant acid phosphatase (TRAP) and immunohistochemical analysis

Femoral bone tissues were obtained from each group. After fixing in 4% paraformaldehyde in PBS at 4 °C for 24 h, femoral bones were decalcified in 15% EDTA decalcifying solution on a shaker for 2 weeks. The decalcified and dehydrated femoral bone tissues were then embedded in paraffin and cut into 3 μm sections. Hematoxylin and Eosin (H&E) staining was performed as previously described [24] and TRAP staining was conducted according to the manufacturer's instructions.

Immunohistochemical analysis was carried out using an antibody against mouse osteocalcin (ab93876, 1:200, Abcam, Cambridge, UK). All sections were observed and photographed on an Olympus BX51 microscope. TRAP and immunohistochemical staining was evaluated by counting the number of stained cells. Osteoblasts/osteoclasts on the bone surface were discerned by morphology and calculated by two independent observers blinded to the groups as described previously [24].

## 2.9. Cell culture

The mouse preosteoblastic cell line MC3T3-E1, human prostate cancer cell lines PC-3 and LNCap, *Rattus norvegicus* kidney cell line NRK-52e, mouse testis sertoli cell line TM-4, mouse chondrogenic cells ATDC5 and human embryonic kidney cell line HEK293 were maintained in Alpha Minimum Essential Medium (α-MEM, Dow Corning Corp., Midland, MI, USA) or Dulbecco's Modified Eagle Medium (DMEM, Dow Corning) supplemented with 10% FBS (Dow Corning), 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate, at 37 °C with 5% CO<sub>2</sub>. For citrate secretion analysis, cells were seeded in 24-well plates at a density of 2.5 × 10<sup>5</sup> cells/well.

Primary osteoblasts/chondrocytes were prepared from the calvaria/costicartilage of 3-day-old neonatal mouse pups as described previously [25, 26] and cultured as described above. Mesenchymal stem cells (MSCs) were isolated from the bone marrow of 8-week-old C57/BL6

mice using standard protocols. Briefly, cells were washed twice with sterile PBS and seeded into six-well plates. After 3 days, non-adherent cells were removed, and the remaining adherent cells expanded until they reached 90% confluence and then used at passage 3 [25].

For osteogenic differentiation, MC3T3-E1 cells and bone mesenchymal stem cells (BMSCs) were seeded into 24-well plate at a density of 2.5 × 10<sup>5</sup> cells/well and induced to differentiate with complete medium supplemented with 100 μg/mL L-ascorbate phosphate (Sigma-Aldrich, St Louis, MO, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich) [27]. Cell supernatant was collected at various time-points (0, 7 and 15 days) for citrate content analysis and cells were lysed and collected for Western blotting or aconitase activity analysis. Those experiments were repeated for at least 3 times. To eliminate the erratic readings caused by proteins in FBS and pyruvate, citrate content in cell supernatant was evaluated in cells incubated with medium without FBS and pyruvate sodium for 6 h. Aconitase activity was determined using aconitase activity colorimetric assay kit (K716–100; Biovision) according to the manufacturer's instructions.

## 2.10. Western blotting

Protein extracts from cells were prepared in 2 × SDS lysis buffer containing β-mercaptoethanol. Protein extracts were subjected to 10% SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes [24]. Then, the membranes were blocked with 5% non-fat milk (w/v, in Tris Buffered Saline (TBS) containing 0.1% Tween 20) and incubated with specific primary antibodies, including anti-citrate synthase (1:4000), anti-ZIP1 (1:1000), anti-CTP (1:2000), anti-Runx2 (1:2000) and anti-α-tubulin (1:8000). After overnight incubation, the membranes were incubated with corresponding secondary antibodies at room temperature for 1 h and visualized by enhanced chemiluminescence.

## 2.11. Statistical analysis

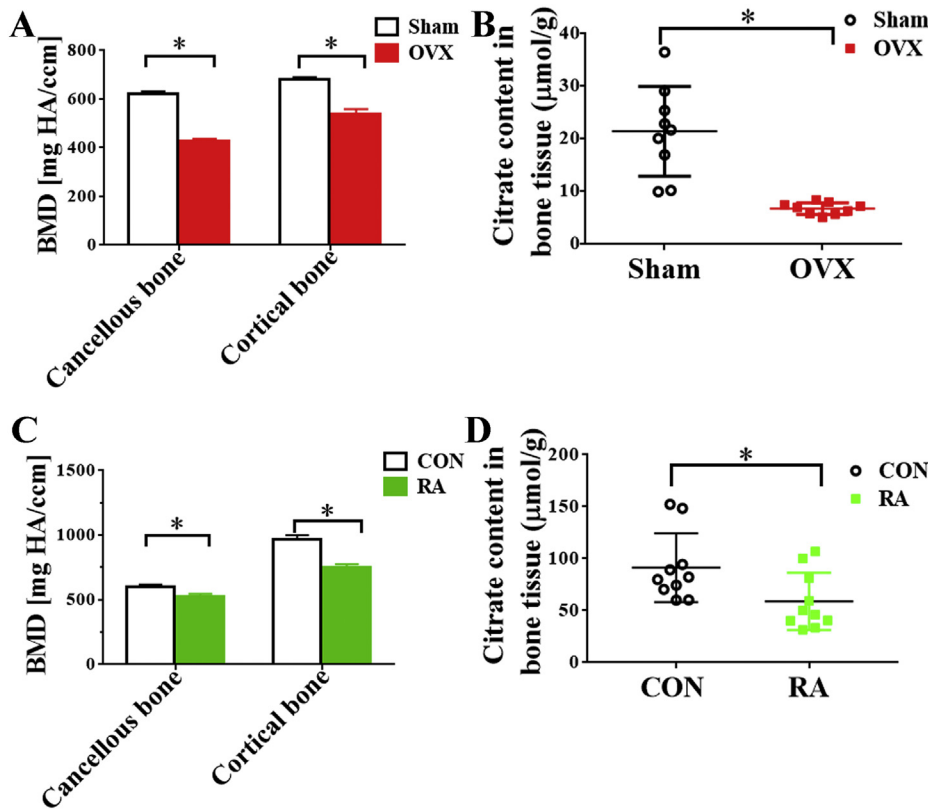
Data were analyzed using SPSS software (SPSS 20.0, IBM Corp., Armonk, NY, USA). All results are presented as the mean ± SD. The parametric variables were compared using Student's-test or one way ANOVA. Curve analysis was determined using GraphPad Prism 7.0. Partial correlation was used to analyze the relationship between BMD, BMI, gender age, serum OCN, serum PINP, serum β-CTX and citrate. The level of significance was set at *P* < 0.05.

## 3. Results

### 3.1. Citrate content of bone is reduced in osteoporotic mice and rats

In order to identify if the amount of citrate changes in various bone loss-related diseases, we established several osteoporosis animal models. Surgery-induced estrogen loss for 3 months caused a marked atrophy of the uterus (Supplemental Fig. S1A) and a significant increase of body weight in mice (Supplemental Fig. S1B). Quantitative micro-CT analysis of histomorphometric parameters of the distal femur showed a marked decrease in femoral BMD (Fig. 1A), cancellous BV/TV, Tb.N, Tb.Th, and outer perimeter of the cortical bone, as well as a significant increase in Tb.Sp (Supplemental Fig. S1C and D). OVX mice also exhibited thinning and disconnection of trabeculae (Supplemental Fig. S2A), and enhanced bone resorption and bone formation, characterized by significant increments of osteoclast and osteoblast surface and numbers (Supplemental Fig. S2B and C). Femora and tibiae of Sham and OVX mice were dissected and subjected to citrate content analysis as described in Materials and methods. Interestingly, citrate content per gram of bone tissue from OVX mice was markedly reduced compared with that of Sham control mice (Fig. 1B).

We further assessed bone citrate content in retinoic acid (RA)-induced osteoporotic rats. Micro-CT analysis demonstrated significant



**Fig. 1.** Citrate content in bone is reduced in osteoporotic mice and rats. (A) Bone mineral density (BMD) of cancellous bone and cortical bone of OVX and Sham mice ( $*P < 0.05$ ). (B) Citrate content in bone of OVX and Sham mice were determined. Citrate content per gram of bone tissue from OVX mice was markedly reduced ( $*P < 0.05$ ). (C) Cancellous and cortical BMD of retinoic acid (RA) treated rats and control rats (CON). RA treated rats showed markedly reduced BMD. (D) Citrate content determination. Citrate content per gram of bone tissue from RA-treated rats contained markedly reduced citrate. Error bars in bar graphs are SEM.  $*P < 0.05$ ,  $n = 10$  for each group.

loss of bone density in the tibiae and femora of retinoic acid-treated rats (Fig. 1C). Consistent with the OVX mice, the bone citrate content was significantly reduced in these osteoporotic rats (Fig. 1D). Taken together, these results demonstrate that, concomitant with bone loss, citrate content in bone is markedly decreased in osteoporotic mice and rats.

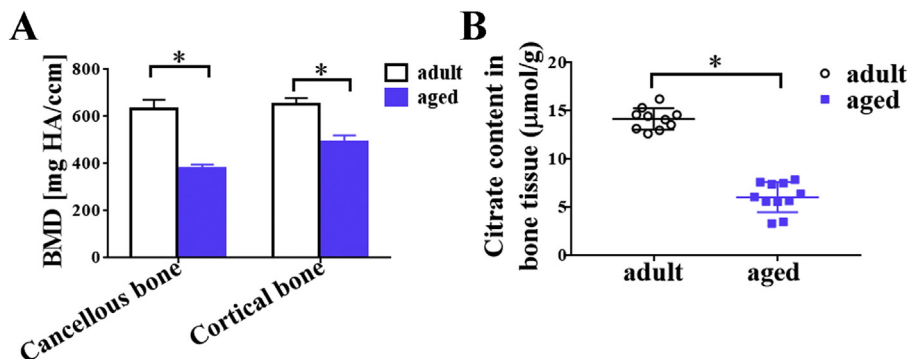
**3.2. Citrate content in bone is reduced in aged mice**

Senile osteoporosis accounts for 20% of cases of primary osteoporosis and is mostly associated with cortical bone, predisposing older women and men to hip fractures [28, 29]. We sought to determine whether the citrate level in bone is reduced during age-related bone loss. Femora from aged (20-month-old) and adult (6-month-old) mice were subjected to micro-CT analysis (Fig. 2A). The aged mice exhibited histopathological findings of osteoporosis, including significant thinning of cortical bone and pronounced diminutions of trabecular bone (Supplemental Fig. S3A and B). The numbers of osteoclasts and

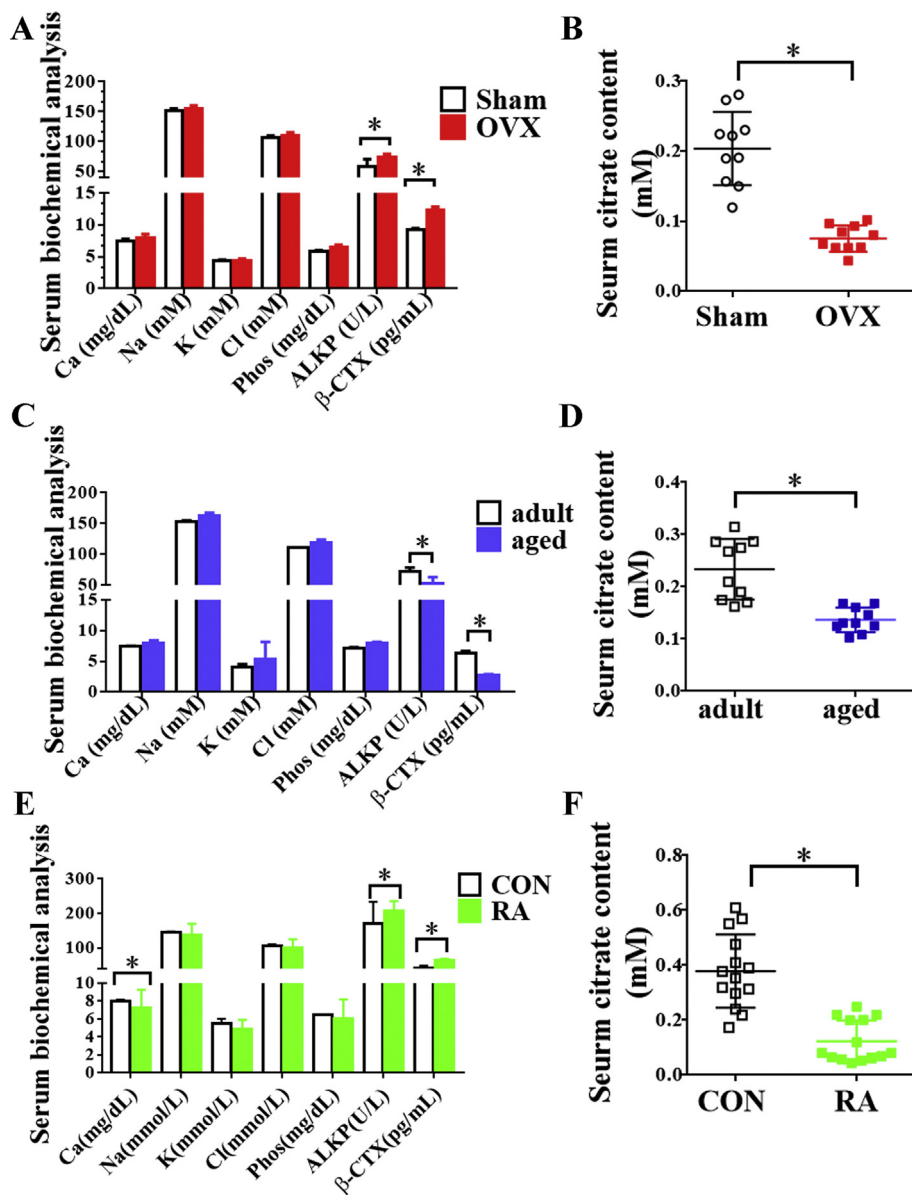
osteoblasts were also dramatically decreased in aged mice, as shown by TRAP staining and osteocalcin staining, respectively (Supplemental Fig. S3C-E). We further evaluated the citrate content in femora and tibiae of adult and aged mice. As expected, there was a significant decrease in the citrate content of bone from senile osteoporotic mice (Fig. 2B). These findings suggest that citrate content in bone decreases with age-related bone loss.

**3.3. Plasmic citrate level is reduced in osteoporotic and aged mice and rats**

Bones are the main storage site of citrate as well as calcium in the body [1], but the role of bone citrate in the homeostatic regulation of citrate is unknown. It is therefore important to evaluate the plasma citrate concentration during age-related as well as estrogen-deficiency-induced bone loss. We first examined serum Ca, Na, K, Cl, Phos, ALKP and  $\beta$ -CTX concentrations in Sham and OVX mice. Apart from an increase in ALKP and  $\beta$ -CTX level, no significant changes of those biochemical parameters were observed in OVX mice compared with those



**Fig. 2.** Citrate content in bone is reduced in aged mice. (A) BMD of cancellous bone and cortical bone of aged mice and adult mice. (B) Citrate content of bones from senile osteoporotic mice showed a significant decrease.  $*P < 0.05$ .



**Fig. 3.** Serum citrate concentration of osteoporotic mice and rats was decreased compared to control. (A, C, E) Serum biochemistry analysis of OVX and Sham mice (A), aged and adult mice (C) and RA treated rats (E). (B, D, F) Serum citrate concentration of OVX and Sham mice (B), aged and adult mice (D) and RA-treated rats (F). \**P* < 0.05, *n* = 10 for each group.

of Sham mice (Fig. 3A). The deproteinized serum was then subjected to citrate analysis. We found that plasmic citrate concentration in OVX mice was significantly lower than that in the Sham group (Fig. 3B). Similar results were found in serum samples of aged mice and retinoic acid-induced osteoporotic rats (Fig. 3C–F). Those results reveal the pathophysiological decrease in plasma citrate concentrations that develops specifically during the process of bone diminution. Bone citrate may be an important component of the homeostatic maintenance of circulating citrate.

**3.4. Plasmic citrate level is reduced in senile osteoporotic patients**

In order to determine whether serum citrate concentration is affected by age-related bone loss in humans, healthy young volunteers (aged at 18.8 ± 0.7) and elderly patients (aged at 64.5 ± 3.9) were recruited and the characteristics of the volunteers and patients are shown in Table 1. Serum citrate concentration in aged males was drastically reduced compared with young healthy males

**Table 1**  
Characteristics of the young volunteers and aged subjects.

	Young (n = 120)	Aged (n = 120)
Age (year)	18.8 ± 0.7 (18–20)	64.5 ± 3.9 (60–70)*
Gender	Male	Male
Height (cm)	173 ± 4	154 ± 9*
Weight (kg)	72.32 ± 9.34	53.76 ± 12.57*
BMI (kg/m <sup>2</sup> )	24.26 ± 5.13	22.66 ± 4.39
Serum citrate (mM)	177.82 ± 98.88	32.1 ± 14.15**
Serum OCN (ng/mL)	16.21 ± 3.12	10.39 ± 4.64*
Serum PINP (ng/mL)	65.23 ± 13.61	71.65 ± 19.28
Serum β-CTX (pg/mL)	923.85 ± 107.25	444.08 ± 348.95**
Fracture history (number)	0	120

\* *P* < 0.05.

\*\* *P* < 0.01, compared with young volunteers.

**Table 2**  
The baseline characteristics of the 87 subjects.

	Male (n = 25)	Female (n = 62)
Age (year)	65.44 ± 12.16	70.61 ± 11.63*
Height (cm)	160.88 ± 9.17	152.27 ± 7.66*
Weight (kg)	57.36 ± 13.66	53.63 ± 11.66
BMI (kg/m <sup>2</sup> )	21.98 ± 3.76	23.18 ± 4.62
Total hip BMD	0.74 ± 0.15	0.61 ± 0.16*
lumbar BMD (L1-L4)	1.07 ± 0.31	0.89 ± 0.2*
Serum citrate (mM)	43.21 ± 15.05	37.80 ± 16.65*
Serum OCN (ng/mL)	9.59 ± 3.26	10.62 ± 4.32
Serum PINP (ng/mL)	43.26 ± 10.23	40.39 ± 9.13
Serum β-CTX (pg/mL)	355.49 ± 48.64	418.74 ± 139.59*
Fracture history (number)	13	26
T score < -2.5 (number)	8	24
-2.5 < T score < -1 (number)	4	19

\*  $P < 0.05$ , compared with male.

(32.1 ± 14.15 mM vs 177.82 ± 98.88 mM). Accordingly, serum osteocalcin levels (10.39 ± 4.64 ng/mL vs 16.21 ± 3.12 ng/mL,  $P < 0.05$ ) and β-CTX (444.08 ± 348.95 pg/mL vs 923.85 ± 107.25 pg/mL,  $P < 0.0001$ ) of aged males were also lower than those of young healthy males.

In order to further analyze the correlation between BMD and plasma citrate, the baseline characteristics of the 87 subjects (25 males and 62 females) are shown in Table 2. Serum citrate level positively correlated with total hip BMD ( $r = 0.457$ ,  $P < 0.001$ , Fig. 4A) and lumbar spine BMD ( $r = 0.610$ ,  $P < 0.001$ , Fig. 4B). However, the association between serum OCN ( $r = 0.246$ ,  $P = 0.054 > 0.05$ ), PINP ( $r = 0.135$ ,  $P > 0.05$ ) or β-CTX ( $r = -0.198$ ,  $P > 0.05$ ) and serum citrate content was not significant (Fig. S4A–C). In addition, no significant difference was found in serum citrate level of different age groups categorized by gender (Table S1).

### 3.5. Osteoblasts are highly specialized cells that produce citrate

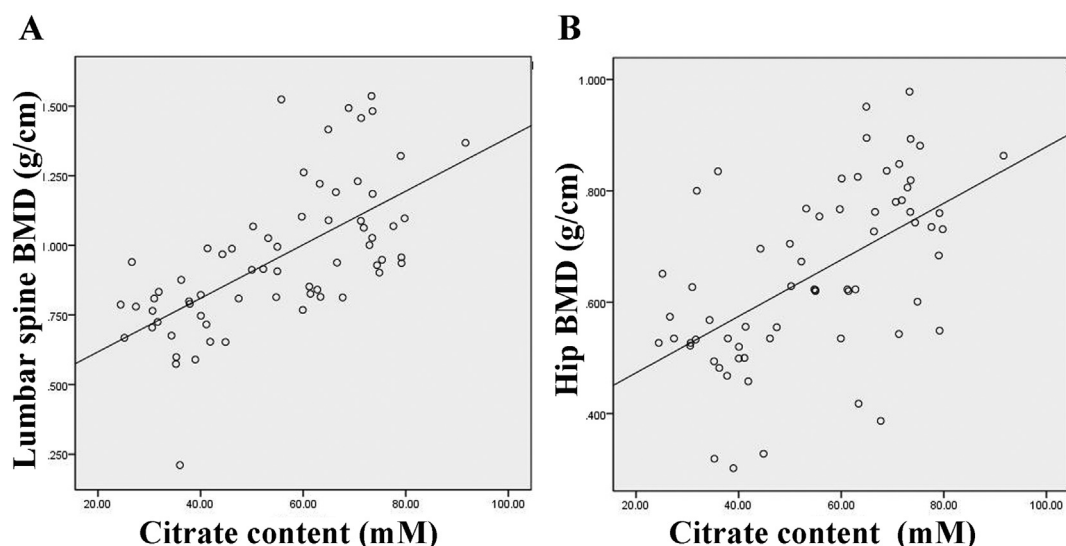
In typical cell metabolism, citrate that is synthesized is essentially utilized by its oxidation *via* the Krebs cycle or as the cytosolic source of acetyl CoA; so that high citrate accumulation does not occur and intracellular citric acid is hardly detectable [30]. It has been shown that prostatic epithelium cells are highly specialized to produce and release high concentrations of citrate during their normal secretory function [31, 32]. *In vitro* differentiated osteoblasts are capable of net citrate

production in contrast to undifferentiated MC3T3-E1 cells [33]. To investigate whether osteoblasts are the main source of citrate in bone, citrate released in supernatants of several cell lines and primary cultured cells were evaluated. As for NRK-52e (rat kidney epithelial cells), TM-4 (mouse sertoli cells), LNCap (prostate cancer cells), HEK293 (human embryonic kidney 293 cells) and ADTC5 (murine chondrogenic cells), citrate production in culture supernatants was barely detectable. However, citrate production was detected in supernatants of PC-3 (prostate cancer cells), MC3T3-E1 cells (osteoblast-like cells), primary osteoblasts and chondrocytes (Fig. 5A). Importantly, citrate in the supernatants increased with the osteoblastic differentiation of BMSC and MC3T3-E1 cells (Fig. 5C and D). Notably, because only 6 h of citrate release from the examined cells was examined as described in Materials and Methods, the measured concentrations of citrate in the culture supernatant were relatively low.

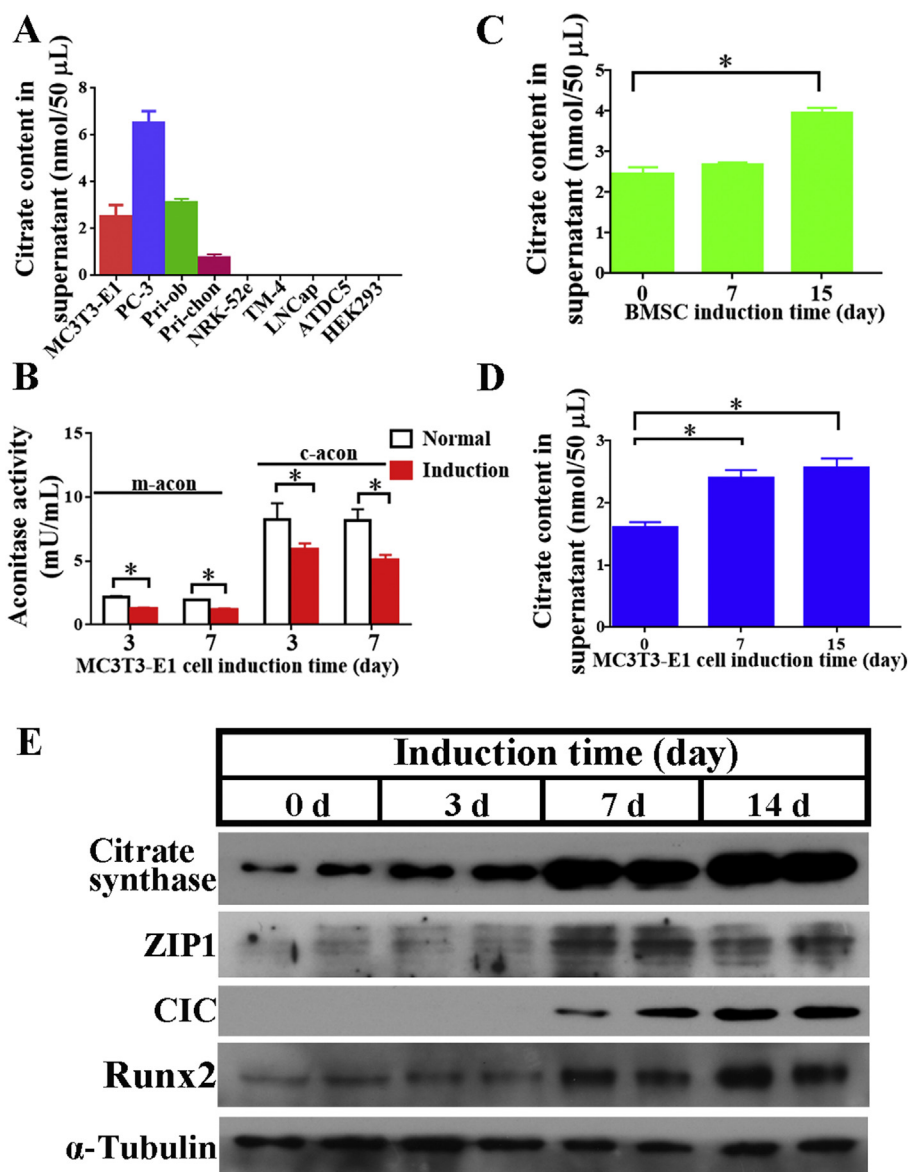
Several proteins involved in the citric acid cycle including SLC39A1 (ZIP1) and mitochondrial aconitase (m-acon) have been shown to play essential roles in net citrate production in prostate epithelial cells [8, 34], while mitochondrial citrate carrier (CIC, SLC25A1) shuttle mitochondrially synthesized citrate to the cytoplasm. To further confirm the citrate production specialty of osteoblasts, the expressions of citrate synthase (CS), CIC and ZIP1 were analyzed during osteoblast differentiation. As shown in Fig. 5E, citrate synthase, CIC and ZIP1 levels were markedly enhanced in differentiated osteoblasts, especially after induction for 7 days. In contrast, m-acon activity was decreased during the osteogenic differentiation of MC3T3-E1 cells, which resulted in reduction of citrate oxidation and increase of citrate secretion (Fig. 5B). These results further support the hypothesis that osteoblasts might be the specialized cells secreting citrate in bone.

## 4. Discussion

Although it has long been known that citrate is accumulated in bone, the origin of the citrate in bone and its role in the development, maintenance and pathology of bone is unknown. In this study, we demonstrate that citrate content is markedly reduced in osteoporotic bone. Plasmic citrate level is also reduced in animals and humans with bone loss, and correlates with lumbar spine and hip BMD in patients with osteoporosis. Osteoblastic cells are capable of producing citrate *in vitro*. Our findings establish a correlation between bone citrate content and osteoporosis, suggesting a critical role of bone citrate in the maintenance of the citrate balance in circulation, and implying that osteoblasts are the highly specialized cells that produce citrate in bone.



**Fig. 4.** Serum citrate content correlated positively with BMD. (A) Positive correlation between serum citrate content and BMD of the lumbar spine ( $r = 0.610$ ,  $P < 0.001$ ). (B) Positive correlation between serum citrate content and BMD of the hip ( $r = 0.457$ ,  $P < 0.001$ ).



**Fig. 5.** Osteoblasts are the specialized cells in bone that produce citrate. (A) Citrate production in supernatants of different cell types. (B) Mitochondrial aconitase (m-acon) and cellular aconitase (c-acon) activity of MC3T3-E1 cells during osteogenic differentiation. Citrate production in cell supernatant during osteoblastic differentiation of BMSCs (C) and MC3T3-E1 cells (D). (E) Western blotting analysis of citrate synthase, ZIP1 and CIC expression during osteoblastic differentiation of MC3T3-E1 cells. Pri-ob, primary osteoblasts. Pri-chon, primary chondrocytes. \* $P < 0.05$ .

Serum citrate level may thus represent a marker for age-related or estrogen deficiency-induced bone loss.

Bone strength is determined by two properties: bone material composition and bone structure design [35, 36]. Since Dickens *et al.* reported the presence of high citrate concentrations in bone tissue in 1941 [1], the special functions of citrate in bone have received considerable attention. By the use of multinuclear magnetic resonance (NMR) analysis and distance measurements, Schmidt-Rohr and colleagues quantitatively analyzed citrate on the bone surface [3]. As a bound component of the apatite nanocomposite-collagen complex, citrate accounts for 5.5 wt% of the organic matter in bone [37, 38]. Davies *et al.* proposed a new structure for bone mineral in which citrate anions in hydrated layers formed bridges between mineral platelets, which can explain a number of known structural features of bone mineral [13]. At the molecular level, strongly bounded citrate molecules are found to form complexes with surface  $\text{Ca}^{2+}$  ions, which significantly decrease the surface hydrophilicity [14]. Moreover, *in vitro* study reveals that high level of citrate can effectively facilitate the intrafibrillar formation

of hydroxyapatite to produce an inorganic-organic composite [15]. Thus, citrate in bone is not a dissolved calcium-solubilizing agent but a strongly bound, integral component of the apatite nanocrystal, which imparts important biomechanical properties and interfacial compatibility to the bone such as its stability, strength, and resistance to fracture [12, 14, 15, 39].

Bone loss as a result of aging and/or estrogen deficiency is the predominant pathophysiologic disorder of primary osteoporosis [20]. Bone fracture is much more frequently seen in osteoporotic patients, especially in aged people of both sex and in post-menopausal women. The proposed structural and functional roles of citrate in bone raised new questions: is the citrate component reduced in osteoporotic bone or is citrate reduction related to bone loss? In this study, we used several osteoporosis animal models including senile, retinoic acid-induced and postmenopausal mice and rats to show that citrate content was markedly reduced in osteoporotic bone. During osteoporosis, an imbalance occurs between bone formation (osteoblasts) and resorption (osteoclasts) as well as an impairment of mesenchymal stem cells (MSC)

commitment towards osteoblasts, in favor of adipocytes [40, 41]. On the one hand, the imbalance between bone formation and resorption may reduce citrate synthesis by osteoblasts. On the other hand, MSC differentiation towards adipocytes requires more citrate as a source of cytosolic acetyl CoA for fatty acid synthesis for lipid biosynthesis [42]. Those pathological conditions linked to osteoporosis may result in a decrease in citrate production in osteoporotic bones, which consequently may reduce citrate incorporation into the structure of bone. The crystallinity of osteoporotic bone mineral and biomechanical properties of bone thus change, which may compromise bone strength and cause fragility fractures in the osteoporotic population. Taken together, although the role of citrate deficiency in osteoporosis remains to be further investigated, our findings suggest that loss of citrate in bone may contribute to osteoporosis-related bone fracture. Notably, there is a difference in bone tissue and serum citrate content between rats and mice. However, the reasons or mechanisms causing the difference between species are yet unclear, further research will be carried out.

It has been long recognized that normal human prostate epithelial cells secrete extraordinarily high levels of citrate [30, 43]. It is achieved by a high intracellular zinc level which can inhibit m-acon activity. Consequently, limited m-acon minimizes citrate oxidation via the Krebs cycle, contributing to citrate accumulation in prostate epithelial cells which is then secreted into human prostate fluid [34, 44, 45]. As in the prostate, bone also contains high levels of zinc along with high citrate levels, but the source of citrate in bone is unclear. Costello *et al.* proposed a concept of “osteoblast citration”, proposing that osteoblasts act as specialized citrate-producing cells during bone formation [33]. In this study, citrate production was observed in osteoblasts but not in many other cells, and its production increased with the osteoblastic differentiation of BMSC and MC3T3-E1 cells. Importantly, we found that the expressions of citrate synthase, ZIP1 and CIC were elevated, while the activity of m-acon was reduced during osteogenic differentiation of MC3T3-E1 cells. Those *in vitro* findings confirm the metabolic changes favoring citrate producing during osteogenic differentiation, suggesting that osteoblasts are highly specialized cells that produce citrate. Further studies are needed to identify whether osteoblasts are responsible for producing bone citrate *in vivo*.

It is well established that zinc has an active role in bone metabolism, and zinc deficiency has been implicated as a risk factor in the development of osteoporosis [46]. Compared to normal controls, zinc levels in serum and bone tissue of patients with senile osteoporosis are lower [47]. Lower serum zinc levels have also been reported in women with postmenopausal osteoporosis [48]. Zn deficiency in osteoporosis may be another reason for the low citrate abundance in osteoporotic bone tissue. Whether zinc replenishment by dietary zinc supplementation would be of value in restoring the production of citrate in bone warrants further study.

Another intriguing finding of this study is that serum citrate level positively correlates with BMD of two specific skeletal regions: lumbar spine and hip. A high correlation between BMD and bone strength, or BMD and fracture risk has been reported [49, 50]. However, BMD at one site might not predict systemic bone strength or fracture risk. We found that a significantly positive correlation between serum citrate level and BMD at two sites, suggesting citrate may be a good marker for bone loss and osteoporotic fracture. Serum citrate level in young healthy males is significantly higher than that of aged males. Serum citrate is also drastically reduced in osteoporotic animals. As indicated above, osteoblasts are the specialized cells in bone that produce citrate. We propose that osteoblasts and bone tissue might act as a citrate pool. Besides incorporating and limiting the size of the apatite nanocrystals, bone citrate may play a critical role in maintaining the citrate balance of the circulation. Serum citrate level may thus represent a marker for bone loss-related diseases such as senile and postmenopausal osteoporosis.

## Declarations of interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2018.06.014>.

## References

- [1] F. Dickens, The citric acid content of animal tissues, with reference to its occurrence in bone and tumour, *Biochem. J.* 35 (1941) 1011–1023.
- [2] E. Seifter, L.S. Lavine, Aspects of citric acid chemistry related to bone, *Bull. N. Y. Acad. Med.* 37 (1961) 156–166.
- [3] H.P. Schwarzk, K. Agur, L.M. Jantz, A new method for determination of postmortem interval: citrate content of bone, *J. Forensic Sci.* 55 (2010) 1516–1522.
- [4] S.J. Wilson, A.M. Christensen, A test of the citrate method of PMI estimation from skeletal remains, *Forensic Sci. Int.* 270 (2017) 70–75.
- [5] A.D. Kenny, P.R. Draskoczy, P. Goldhaber, Citric acid production by resorbing bone in tissue culture, *Am. J. Phys.* 197 (1959) 502–504.
- [6] M. Johnsson, C.F. Richardson, J.D. Sallis, G.H. Nancollas, Adsorption and mineralization effects of citrate and phosphocitrate on hydroxyapatite, *Calcif. Tissue Int.* 49 (1991) 134–137.
- [7] M.L. de Rezende, P.T. Coesta, R.C. de Oliveira, S. Salmeron, A.C. Sant’Ana, C.A. Damante, S.L. Greggi, A. Consolaro, Bone demineralization with citric acid enhances adhesion and spreading of preosteoblasts, *J. Periodontol.* 86 (2015) 146–154.
- [8] L. Costello, R. Franklin, P. Narayan, Citrate in the diagnosis of prostate cancer, *Prostate* 38 (1999) 237–245.
- [9] D.P. Simpson, Citrate excretion: a window on renal metabolism, *Am. J. Phys.* 244 (1983) F223–F234.
- [10] L.C. Costello, R.B. Franklin, Citrate metabolism of normal and malignant prostate epithelial cells, *Urology* 50 (1997) 3–12.
- [11] R. Apsner, M. Schwarzenhofer, K. Derfler, C. Zauner, K. Ratheiser, A. Kranz, Impairment of citrate metabolism in acute hepatic failure, *Wien. Klin. Wochenschr.* 109 (1997) 123–127.
- [12] Y.-Y. Hu, A. Rawal, K. Schmidt-Rohr, Strongly bound citrate stabilizes the apatite nanocrystals in bone, *Proc. Natl. Acad. Sci.* 107 (2010) 22425–22429.
- [13] K.H.M. Erika Davies, Wai Ching Wong, Chris J. Pickard, David G. Reid, Jeremy N. Skepper, Melinda J. Duer, Citrate bridges between mineral platelets in bone, *PNAS* 111 (2014) E1354–E1363.
- [14] P. Ivanchenko, J.M. Delgado-Lopez, M. Iafisco, J. Gomez-Morales, A. Tampieri, G. Martra, Y. Sakhno, On the surface effects of citrates on nano-apatites: evidence of a decreased hydrophilicity, *Sci. Rep.* 7 (2017) 8901.
- [15] C. Shao, R. Zhao, S. Jiang, S. Yao, Z. Wu, B. Jin, Y. Yang, H. Pan, R. Tang, Citrate improves collagen mineralization via interface wetting: a physicochemical understanding of biomineralization control, *Adv. Mater.* 30 (2018).
- [16] Y. Guo, R.T. Tran, D. Xie, Y. Wang, D.Y. Nguyen, E. Gerhard, J. Guo, J. Tang, Z. Zhang, X. Bai, J. Yang, Citrate-based biphasic scaffolds for the repair of large segmental bone defects, *J. Biomed. Mater. Res. A* 103 (2015) 772–781.
- [17] J. Guo, G.B. Kim, D. Shan, J.P. Kim, J. Hu, W. Wang, F.G. Hamad, G. Qian, E.B. Rizk, J. Yang, Click chemistry improved wet adhesion strength of mussel-inspired citrate-based antimicrobial bioadhesives, *Biomaterials* 112 (2017) 275–286.
- [18] D. Shan, C. Zhang, S. Kalaba, N. Mehta, G.B. Kim, Z. Liu, J. Yang, Flexible biodegradable citrate-based polymeric step-index optical fiber, *Biomaterials* 143 (2017) 142–148.
- [19] J. Guo, Z. Xie, R.T. Tran, D. Xie, D. Jin, X. Bai, J. Yang, Click chemistry plays a dual role in biodegradable polymer design, *Adv. Mater.* 26 (2014) 1906–1911.
- [20] J.B. van Meurs, R.A. Dhonukshe-Rutten, S.M. Pluijm, M. van der Klift, R. de Jonge, J. Lindemans, L.C. de Groot, A. Hofman, J.C. Witteman, J.P. van Leeuwen, M.M. Breteler, P. Lips, H.A. Pols, A.G. Uitterlinden, Homocysteine levels and the risk of osteoporotic fracture, *N. Engl. J. Med.* 350 (2004) 2033–2041.
- [21] E.Y. Liao, X.H. Luo, W.B. Wang, X.P. Wu, H.D. Zhou, R.C. Dai, H.J. Liao, C. Yang, Effects of different nylestriol/levonorgestrel dosages on bone metabolism in female Sprague-Dawley rats with retinoic acid-induced osteoporosis, *Endocr. Res.* 29 (2003) 23–42.
- [22] M. Zhang, S. Xuan, M.L. Bouxsein, S.D. Von, N. Akeno, M.C. Faugere, H. Malluche, G. Zhao, C.J. Rosen, A. Efstratiadis, Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization, *J. Biol. Chem.* 277 (2002) 44005–44012.
- [23] L.C. Costello, M. Chellaiah, J. Zou, R.B. Franklin, M.A. Reynolds, The status of citrate in the hydroxyapatite/collagen complex of bone; and its role in bone



- formation, *J. Regen. Med. Tissue Eng.* 3 (2015) 4.
- [24] B. Huang, Y. Wang, W. Wang, J. Chen, P. Lai, Z. Liu, B. Yan, S. Xu, Z. Zhang, C. Zeng, mTORC1 prevents preosteoblast differentiation through the notch signaling pathway, *J. Orthop. Transl.* 11 (2015) e1005426.
- [25] T. Garcia, S. Roman-Roman, A. Jackson, J. Theilhaber, T. Connolly, S. Spinella-Jaegle, S. Kawai, B. Courtois, S. Bushnell, M. Auberval, Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro, *Bone* 31 (2002) 205–211.
- [26] A.J. Miranda, Y. Dong, J. Kim, M.J. Hilton, Isolation and culture of murine primary chondrocytes, *Methods Mol. Biol.* 1130 (2014) 267.
- [27] J.B. Park, The effects of dexamethasone, ascorbic acid, and  $\beta$ -glycerophosphate on osteoblastic differentiation by regulating estrogen receptor and osteopontin expression, *J. Surg. Res.* 173 (2012) 99–104.
- [28] A. Wawrzyniak, W. Horstikorska, Senile osteoporosis, *Pol. Arch. Med. Wewn.* 118 (2008) 59 (Suppl).
- [29] G.D.M. Phd, B.R. Troen, Understanding the mechanisms of senile osteoporosis: new facts for a major geriatric syndrome, *J. Am. Geriatr. Soc.* 56 (2008) 935–941.
- [30] L.C. Costello, R.B. Franklin, Concepts of citrate production and secretion by prostate. 1. Metabolic relationships, *Prostate* 18 (1991) 25–46.
- [31] J.P. Kavanagh, Isocitric and citric acid in human prostatic and seminal fluid: implications for prostatic metabolism and secretion, *Prostate* 24 (1994) 139–142.
- [32] L.C. Costello, R.B. Franklin, The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy, *Oncology* 59 (2000) 269–282.
- [33] R.B. Franklin, M. Chellaiah, J. Zou, M.A. Reynolds, L.C. Costello, Evidence that osteoblasts are specialized citrate-producing cells that provide the citrate for incorporation into the structure of bone, *Open Bone J.* 6 (2014) 1–7.
- [34] L.C. Costello, Y. Liu, R.B. Franklin, M.C. Kennedy, Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells, *J. Biol. Chem.* 272 (1997) 28875–28881.
- [35] H.K. Datta, W.F. Ng, J.A. Walker, S.P. Tuck, S.S. Varanasi, The cell biology of bone metabolism, *J. Clin. Pathol.* 61 (2008) 577.
- [36] E. Seeman, P.D. Delmas, Bone quality—the material and structural basis of bone strength and fragility, *N. Engl. J. Med.* 26 (2008) 1.
- [37] Y. Wu, J.L. Ackerman, H.M. Kim, C. Rey, A. Barroug, M.J. Glimcher, Nuclear magnetic resonance spin-spin relaxation of the crystals of bone, dental enamel, and synthetic hydroxyapatites, *J. Bone Miner. Res.* 17 (2002) 472–480.
- [38] C. Jager, T. Welzel, W. Meyer-Zaika, M. Epple, A solid-state NMR investigation of the structure of nanocrystalline hydroxyapatite, *Magn. Reson. Chem.* 44 (2006) 573–580.
- [39] B. Xie, G.H. Nancollas, How to control the size and morphology of apatite nanocrystals in bone, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 22369–22370.
- [40] J.W. Zhao, Z.L. Gao, H. Mei, Y.L. Li, Y. Wang, Differentiation of human mesenchymal stem cells: the potential mechanism for estrogen-induced preferential osteoblast versus adipocyte differentiation, *Am J Med Sci* 341 (2011) 460–468.
- [41] K. Matsuda, Y. Ito, Y. Kanemitsu, Osteoblasts and adipocytes: human skeletal (mesenchymal) stem cells lineage fate regulation, *Bone* 47 (2010) S16–S17.
- [42] J.A. Watson, J.M. Lowenstein, Citrate and the conversion of carbohydrate into fat. Fatty acid synthesis by a combination of cytoplasm and mitochondria, *J. Biol. Chem.* 245 (1970) 5993.
- [43] L.C. Costello, R.B. Franklin, Prostate epithelial cells utilize glucose and aspartate as the carbon sources for net citrate production, *Prostate* 15 (1989) 335–342.
- [44] L.C. Costello, R.B. Franklin, Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer, *Prostate* 35 (1998) 285–296.
- [45] N. Dubi, L. Gheber, D. Fishman, I. Sekler, M. Hershinkel, Extracellular zinc and zinc-citrate, acting through a putative zinc-sensing receptor, regulate growth and survival of prostate cancer cells, *Carcinogenesis* 29 (2008) 1692–1700.
- [46] J. Eberle, S. Schmidmayer, R.G. Erben, M. Stangassinger, H.P. Roth, Skeletal effects of zinc deficiency in growing rats, *J. Trace Elem. Med. Biol.* 13 (1999) 21–26.
- [47] O.S. Atik, Zinc and senile osteoporosis, *J. Am. Geriatr. Soc.* 31 (1983) 790–791.
- [48] A. Gur, L. Colpan, K. Nas, R. Cevik, J. Sarac, F. Erdogan, M.Z. Duz, The role of trace minerals in the pathogenesis of postmenopausal osteoporosis and a new effect of calcitonin, *J. Bone Miner. Metab.* 20 (2002) 39–43.
- [49] H.K. Genant, K. Engelke, T. Fuerst, C.C. Gluer, S. Grampp, S.T. Harris, M. Jergas, T. Lang, Y. Lu, S. Majumdar, A. Mathur, M. Takada, Noninvasive assessment of bone mineral and structure: state of the art, *J. Bone Miner. Res.* 11 (1996) 707–730.
- [50] F. Eckstein, E.M. Lochmuller, C.A. Lill, V. Kuhn, E. Schneider, G. Delling, R. Muller, Bone strength at clinically relevant sites displays substantial heterogeneity and is best predicted from site-specific bone densitometry, *J. Bone Miner. Res.* 17 (2002) 162–171.