

# Plasma exosomes derived from patients with end-stage renal disease and renal transplant recipients have different effects on vascular calcification

**xiao lin**

the second xiangya hospital, central south university <https://orcid.org/0000-0002-6039-3630>

**Ting Zhu**

central south university

**Feng Xu**

central south university

**Jia-Yu Zhong**

central south university

**Fuxingzi Li**

central south university

**Su-Kang Shan**

central south university

**Feng Wu**

central south university

**Bei Guo**

central south university

**Ming-Hui Zheng**

central south university

**Yi Wang**

central south university

**Qiu-Shuang Xu**

central south university

**Xiao-Bo Liao**

central south university

**Xu-Biao Xie**

central south university

**Ling-Qing Yuan** (✉ [allenylq@csu.edu.cn](mailto:allenylq@csu.edu.cn))

<https://orcid.org/0000-0001-8602-7113>

**Keywords:** ESRD, Exosomes, Vascular calcification, Fetuin-A, MGP, Annexin-A2

**Posted Date:** July 16th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-41895/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

End-stage renal disease (ESRD) patients usually develop extensive and progressive vascular calcification, and lots of calcification inhibitors as well as pro-calcifying factors are involved in the process. However, the mechanisms of vascular calcification in ESRD patients are still ill-defined.

## Methods

The participants in the study were selected at the Second Xiangya Hospital, Central South University, and the plasma exosomes derived from ESRD patients (ESRD-Ex), renal transplant recipients (RTR-Ex), and the normal health control group (Nor-Ex) were isolated by ExoQuick-TC kit. Transmission electron microscopy and molecular size analysis were used to assess the morphology and size of exosomes. Alizarin Red S staining was carried out to detect calcification of vascular smooth muscle cells (VSMCs). Protein levels of Fetuin-A, matrix gla protein (MGP), Annexin-A2, bone morphogenetic protein (BMP-2), and receptor activator for nuclear factor- $\kappa$  B ligand (Rankl) were measured by Western Blot analysis and the contents of that were detected using ELISA. Coronary artery calcification scores (CACS) were quantified via Agaston and analysed by Siemens CaScoring software.

## Results

Compared with Nor-Ex, the ESRD-Ex promoted calcification of VSMCs significantly, and RTR-Ex could attenuate VSMCs calcification. Moreover, the protein concentration of ESRD-Ex was significantly higher than Nor-Ex and RTR-Ex, and the content of both MGP and Fetuin-A, the calcification inhibitors, were prominently lower in ESRD-Ex than those in Nor-Ex. The content of Annexin-A2, one of the calcification promoters, was significantly higher in ESRD-Ex and RTR-Ex than that in Nor-Ex. But, BMP-2 and Rankl had no significant difference among the three groups. In addition, the content of Fetuin-A in RTR-Ex was higher than that in ESRD-Ex, though it was still lower than in Nor-Ex. Furthermore, the content of both plasma Fetuin-A and MGP were negatively while that of Annexin-A2 was negatively correlated to CACS.

## Conclusions

These results indicated that ESRD-Ex significantly promoted VSMCs calcification while renal transplantation could partially attenuate the effect of exosomes. Fetuin-A and MGP were decreased but Annexin-A2 was increased in ESRD-Ex, and renal transplantation could increase the level of Fetuin-A rather than MGP.

## Introduction

Vascular calcification is mainly characterized as the medial of aortic calcification (termed as Mönckeberg's calcification), and it often happens in patients with chronic kidney disease, especially end stage renal disease (ESRD)[1, 2]. Lots of studies have demonstrated that vascular calcification is not a simple passive process of calcium and phosphorus deposition but an active regulatory process, and the transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells is regarded to be the key pathophysiological factor of vascular calcification [3–5]. However, the specific mechanism of vascular calcification in patients with ESRD is still not entirely elucidated.

Exosomes (Ex) are of endosomal origin from multivesicular bodies. They have a diameter of 30–100 nm, and they are released into the extracellular matrix. Numerous studies have reported that exosomes are usually regarded as mediator of cell to cell communication in physiological and pathological conditions, because of their variety and the abundance of specific cargos including proteins, lipids, and nucleic acids[6–8]. Recently, increasing evidence has shown that exosomes also play an important role in regulating vascular calcification [9, 10]. For instance Li et al. reported that exosomes derived from high glucose induced endothelial cells could carry versican protein to accelerate VSMCs calcification [9]. Our previous study demonstrated that melatonin alleviated vascular calcification through an exosomal miR-204/miR-211 cluster in a paracrine manner [11]. However, the role and mechanism of exosomes in regulating vascular calcification in patients with ESRD is not very clear.

Vascular calcification is an active regulatory process, and a lot of calcification inhibitors as well as pro-calcifying factors are involved [12, 13]. Numerous studies have confirmed that Fetuin-A and matrix gla protein (MGP) are well known calcification inhibitors, and some procalcifying factors, including bone morphogenetic protein (BMP-2), receptor activator of nuclear factor- $\kappa$ B ligand (Rankl), and Annexin-A2, are involved in regulating vascular calcification [12, 13]. When BMP-2 was increased in uremic serum, it could enhance VSMCs calcification *in vitro* [14], and Rankl promoted osteoblastic differentiation of VSMCs by promoting endothelial to release BMP-2 [15]. In addition, studies demonstrated that Annexin-A2 was significantly increased in calcified bovine vascular smooth muscle cells [16], and inhibition of annexin activity decreased chondrocyte mineralization [17]. Herein, it is urgent to investigate whether the calcification related factors are involved in exosomes regulating vascular calcification in patients with ESRD.

In the present study, we found that plasma exosomes derived from patients with ESRD promote VSMCs calcification, while plasma exosomes from renal transplantation patients could partly attenuate VSMCs calcification. The mechanism study demonstrated that the content of calcification inhibitors (Fetuin-A and MGP) decreased significantly, while Annexin-A2, one of procalcifying factors, increased greatly in both exosomes and circulating blood derived from patients with ESRD. On the other hand, renal transplantation could partially restore the level of Fetuin-A in plasma exosomes and then attenuate the vascular calcification.

## Results

## **Vascular calcification is associated with calcium and phosphorus metabolism disorders in patients with ESRD**

Twenty four participants were involved in this study, and the ages of the participants ranged from 25 to 56 years old. The course of the ESRD (starting from the time of diagnosis of ESRD) group ranged from 0.42 to 6 years, with an average course of 3.12 years. These patients included 2 patients with peritoneal dialysis and 6 patients with hemodialysis. The course of the RTR (starting from the time of renal transplantation for patients with ESRD group ranged from 0.33 to 5.5 years, with an average course of 1.9 years. Among them, 3 patients underwent peritoneal dialysis and 5 patients underwent hemodialysis before renal transplantation. The clinical characteristics of the participants are shown in Table 1.

The results showed that the distribution of age had no statistical difference among the three groups, and there were also no significant differences in triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), or low density lipoprotein (LDL) among the three groups. But the serum creatinine (Cre) and blood urea nitrogen (BUN) had statistically significant differences among the three groups. Compared with the normal health control, the levels of corrected serum calcium (csCa), phosphorus (P), Ca×P product, and parathyroid hormone (PTH) in patients with ESRD were significantly increased. However, P, Ca×P, and PTH decreased significantly in the RTR group when compared with the ESRD group. However, there was no significant difference in csCa between ESRD and RTR groups. Besides, the blood level of 25(OH)D in patients with ESRD was much lower than that in a normal healthy control, and it was a slightly increased in patients with RTR, but there was no significant difference between the ESRD group and RTR group. These results suggested that vascular calcification might be related to high Ca, high P, high PTH, and low 25(OH)D levels in patients with ESRD, and renal transplantation may attenuate vascular calcification via decreasing the P and PTH rather than Ca and 25(OH)D.

### **Identification of exosomes**

Exosomes were isolated from the plasma of normal health (Nor-Ex), ESRD patients (ESRD-Ex), and RTR patients (RTR-Ex). The transmission electron microscopy showed that the exosomes displayed a bilayer structure morphology, and the mean diameter detected by a molecular size analyser was  $85.5 \pm 3.96$  nm (Figure 1A and 1B). Western Blot analysis further verified that the presence of the exosome markers, including CD9, CD63 and CD81, was increased in ESRD-Ex and RTR-Ex when compared with Nor-Ex (Figure 1C). All of these features confirmed that the vesicles were actually exosomes.

### **ESRD-Ex promote VSMCs calcification, while RTR-Ex inhibit VSMCs calcification**

To explore the mechanism involved in the vascular calcification, we used the exosomes isolated from the plasma of normal health (Nor-Ex), ESRD patients (ESRD-Ex), and RTR patients (RTR-Ex) to treat VSMCs and observed the calcification of the VSMCs. The fluorescence microscopy analysis revealed that PKH26-labeled exosomes could be taken up and incorporated into VSMCs (Figure 2A). When VSMCs were cultured with Nor-Ex, ESRD-Ex, and RTR-Ex, we found that both ESRD-Ex and RTR-Ex could enhance VSMCs calcification by increasing mineralized nodule formation and Runx2 expression, while Nor-Ex

could inhibit VSMCs calcification. However, mineralized nodule formation and the level of Runx2 were decreased in VSMCs treated with RTR-Exo more than with ESRD-Ex (Figure 2B and 2C). These data suggested that ESRD-Ex promoted VSMCs calcification significantly, while renal transplantation could partially attenuate the effect of exosomes.

### **The content of calcification inhibitors were decreased in ESRD-Ex**

To further explore the mechanisms of ESRD-Ex regulating VSMCs calcification, we determined the calcification promoter and inhibitor in Nor-Ex, ESRD-Ex, and RTR-Ex. Previous studies have demonstrated that Fetuin-A and MGP are calcification inhibitors. Our results showed that the protein concentration of ESRD-Ex and RTR-Ex were much higher than that of Nor-Ex, though the protein concentration of RTR-Ex was lower than that of ESRD-Ex (Figure 3A). The ELISA results further showed that the content of Fetuin-A was the highest in Nor-Ex and decreased significantly in ESRD-Ex. The content of Fetuin-A was increased in RTR-Ex, although it remained lower than that in Nor-Ex (Figure 3B). Besides, the content of MGP in both ESRD-Ex and RTR-Ex were lower than that in Nor-Ex, but the levels of MGP between ESRD-Ex and RTR-Ex had no significant difference (Figure 3C). In addition, Western Blot verified the same results as the ELISA (Figure 3D). Coronary artery calcification is an important form of vascular calcification, and the coronary artery calcification score (CACS) is often used to evaluate the severity of vascular calcification. CACS showed that the mean CACS in ESRD patients was  $313.9 \pm 204.98$ , and it was reduced significantly in patients with RTR (Figure 3E). Furthermore, the Spearman analysis showed that the content of both Fetuin-A and MGP in plasma were negatively correlated to CACS (Figure 3F and 3G). These data demonstrated that Fetuin-A and MGP were decreased in ESRD-Ex, and renal transplantation could partially increase the level of Fetuin-A rather than MGP in the exosomes.

### **Calcification promoter Annexin-A2 was increased in ESRD-Ex**

Calcification promoters are another kind of important factor that affect the progress of vascular calcification, and a previous study showed that Annexin-A2, BMP-2, and Rankl are calcification promoters. In the present study, we found that the content of Annexin-A2 in both ESRD-Ex and RTR-Ex were higher than that in Nor-Ex, but there was no difference in the levels of Annexin-A2 between ESRD-Ex and RTR-Ex (Figure 4A). Nevertheless, the content of both BMP-2 and Rankl had no significant difference among the three groups (Figure 4B and 4C). Besides, Western Blot analyses showed results similar to the ELISA tests (Figure 4D). Meanwhile, the content of Annexin-A2 in plasma was positively related to CACS (Figure 4E), while that of BMP-2 and Rankl had no significant correlation with CACS (Figure 4F and 4G). These results demonstrated that it was Annexin-A2, rather than BMP-2 and Rankl, that was increased in ESRD-Ex, and renal transplantation had no significant effect on the contents of Annexin-A2, BMP-2, and Rankl.

## **Discussion**

In the present study, we clarified that ESRD-Ex promoted VSMCs calcification significantly while renal transplantation could partially attenuate the effect of exosomes. The mechanism study found that

Fetuin-A and MGP were decreased, but Annexin-A2 was increased in ESRD-Ex. Moreover, renal transplantation could increase the level of Fetuin-A rather than MGP, but the contents of Annexin-A2, BMP-2 and Rankl were not affected. In addition, we found that the content of both plasma Fetuin-A and MGP were negatively while that of Annexin-A2 was positively correlated to CACS.

Vascular calcification is common in patients with ESRD, and the related cardiovascular complications are the main causes of mortality in patients with ESRD. Renal replacement therapy including hemodialysis, peritoneal dialysis, and renal transplantation are the main treatments for patients with ESRD [2]. Vascular medial calcification is mainly contributed to calcium and phosphate deposition [5, 18]. In this study, there was no significant difference in blood lipid among patients with ESRD, RTR, and the healthy control. Blood calcium usually refers to the calcium ion in plasma and blood phosphorus refers to the plasma inorganic phosphorus. csCa was used in our study because serum calcium was affected by the level of serum albumin concentration. Under physiological conditions, the concentration of serum calcium and phosphorus is kept in balance, and the  $Ca \times P$  product is usually used as an indicator of osteogenesis [19]. In our present study, the csCa, P, and the calcium-phosphorus product were significantly higher in ESRD patients than in healthy controls. Though there is no significant difference in the csCa between ESRD and RTR patients, the P and calcium-phosphorus product was decreased greatly in RTR patients and had no significant difference when compared with that in the normal health control. These data suggested that calcium and phosphorus metabolism disorder is one of the mechanisms to promote VC in patients with ESRD. In addition, 25(OH)D reflects the level of vitamin D in the body, and a decrease in that can promote vascular medial calcification and contribute to the high mortality in patients with ESRD [20]. Accordingly, our study is consistent with previous studies and further demonstrates that renal transplantation can improve calcium and phosphorus metabolism disorders in patients with ESRD. Therefore, reducing the high calcium and phosphorus load as well as reasonable supplement of vitamin D before kidney transplantation can help to reduce vascular medial calcification.

Exosomes are an extracellular vesicle that can be produced by various types of cells and serve as an important participant in intercellular communication by transporting a variety of bioactive materials (proteins, nucleic acids, etc.) to other cells under physiological and pathological conditions [7, 10]. Recently, extracellular vesicle induced vascular medial calcification was one of the mechanisms of accelerated vascular calcification in patients with chronic kidney disease [19]. Accordingly, our results showed that the protein concentrations of ESRD-Ex were higher than Nor-Ex. Besides, the expression of Runx2 and mineralized nodules were increased significantly in VSMCs treated with ESRD-Ex. These data suggested that plasma exosomes were involved in the regulation of vascular calcification.

Increasing evidence has shown that MGP, Fetuin-A, Rankl, BMP2, Annexin-A2, and other molecules are related to vascular calcification, and the level of calcification promoters is often increased and accompanied by a decrease in calcification inhibitors in patients with chronic kidney disease [16, 21, 22]. MGP is an important calcification inhibitor and it was significantly down-regulated in the calcified arteries [21]. Recent studies further proved that high levels of inactive MGP, also known as uncarboxylated MGP (ucMGP), were significantly correlated with vascular medial calcification in patients with chronic kidney

disease [21, 23]. At the same time, Sipanan Thamratnopkoon showed that the level of ucMGP was increased with the progressive disease of chronic kidney disease [24]. However, the blood concentration of ucMGP depends on the nutritional status of human vitamin K [21, 25]. In order to avoid the effects of vitamin K, the total content of MGP, including ucMGP and carboxylated MGP, was detected in our present study, and the results showed that the plasma total MGP was much lower in ESRD-Ex and RTR-Ex compared with that in the Nor-Ex. Fetuin-A is another key calcification inhibitor, and it inhibits the formation and precipitation of apatite precursors by enhancing the expression of MGP [26]. Many studies have confirmed that low levels of Fetuin-A are related to vascular calcification, and it is an independent risk factor for cardiovascular events in patients with ESRD [22, 27]. Consistent with these previous research results, our present study showed that the plasma Fetuin-A and total MGP were much lower in both ESRD-Ex and RTR-Ex.

In addition, elevated serum BMP-2 in patients with chronic kidney disease induced VSMCs calcification, and Rankl could promote vascular calcification by inducing the release of BMP-2 by vascular endothelial cells.[14, 15] Besides, Annexin-A2 could bind to Fetuin-A at the cell membrane of VSMCs in the presence of high calcium and contribute to SMCs and osteoblast-derived matrix vesicle mediated calcification [16, 28]. Accordingly, our results demonstrated that the levels of Annexin-A2 were much higher in ESRD-Ex and RTR-Ex than that in Nor-Ex, but the concentration of Rankl and BMP-2 had no difference among them. CAC is highly prevalent and more severe in patients with ESRD, and it independently predicts the risk of coronary heart disease and total cardiovascular disease [29]. Interestingly, we found that the content of both Fetuin-A and MGP in plasma were negatively while that of Annexin-A2 was positively related to CACS. Therefore, our experiment demonstrated that the lower levels of plasma MGP and Fetuin-A but the high level of Annexin-A2 might be the one of the reasons that ESRD-Ex promotes VSMCs calcification.

However, there are important limitations of this study that are worth mentioning. Firstly, only 8 subjects were included in each group, and it is necessary to expand the samples to enhance the power of our analyses. Secondly, the origin of exosomes and the bioactive materials carried by exosomes to regulate VSMCs calcification need further study. Thirdly, the molecular mechanism of modulating the levels of calcification related factors (MGP, Fetuin-A, and Annexin-A2) was not clarified in this study. Last but not the least, it is very important to apply the exosomes to model animals and follow up further on the situation of vascular calcification in patients with renal transplant.

In summary, our results demonstrate that the disorders of calcium and phosphorus metabolism and the contents of plasma exosomes are important factors that trigger vascular calcification in patients with ESRD. Moreover, the decrease of calcification inhibitors and the increase of some calcification promoters in plasma exosomes play a key role in the process of vascular calcification in patients with ESRD. Interestingly, renal transplantation could partially attenuate vascular calcification. Therefore, the present study provides new insights for preventing and treating vascular calcification and hope for improving the quality of life and reducing medical costs for patients with ESRD.

## Materials And Methods

## Patients and specimens

A total of 8 patients with stage 5 chronic kidney disease in the department of nephrology and 8 patients who underwent postoperative renal transplantation re-examination in the department of the Center of Organ Transplantation during the same period were selected at the Second Xiangya Hospital, Central South University. Meanwhile, 8 healthy age paired adult volunteers in the physical examination centre were recruited in this study. They were divided into three groups: ESRD, RTR, and normal healthy controls (Nor). The inclusion criteria were as follows: chronic kidney disease patients with the glomerular filtration rate (GFR) less than 15 ml/min/1.73 m<sup>2</sup> of body-surface areas were selected as the ESRD group. Patients with renal transplantation due to ESRD but whose renal function was normal were enrolled into the RTR group. The healthy volunteers with normal renal function and without other diseases such as a malignant tumour, hypertension, diabetes, coronary heart disease, and so on were enrolled into the Normal group. The clinical parameters of all the participants in this study are presented in Table 1.

## Coronary artery calcification measurement

Coronary artery calcification scores (CACS) were calculated using the Siemens Somatom Definition computed tomographic multi-layer spiral scanner (Germany) and the calcification of coronary arteries was quantified via Agaston and analysed by Siemens CaScoring software (syngo. via, Siemens Healthcare GmbH). The width of the detector was 128×0.6 mm and the scan thickness was 1.5 mm simultaneously over 50-70 images of fault scans. A total coronary artery calcification score was generated by using the Agatston method, which has been described before.[30] The vessels evaluated included the left main coronary artery (LM), left anterior descending coronary artery (LAD), left circumflex (CX), and the right coronary artery (RCA). The measure of the area of calcification lesions times a fixed coefficient (the maximum pixel density decision) and the total score of the calcification of all faults was termed the CACS for the patient.

## Exosome extraction

Blood was obtained from patients with ESRD, patients with RTR, and the Normal group, respectively. The blood was centrifuged at 3000×g for 20 min and the supernatant plasma was subjected to the ExoQuick-TC Exosome Precipitation Solution Kit (System Biosciences, USA) for exosomes extraction. Briefly, 250 ul of plasma was added to 63ul ExoQuick Exosomes Precipitation Reagent and fully mixed. After the mixture had been incubated at 4 °C for 30 min, it was centrifuged at 1500× g for 30 min at room temperature, and the yellow or white precipitate in the bottom of the EP tube was the exosomes. Then, the supernatant was removed by aspiration and the exosome pellets were centrifuged again at 1500× g for 5 min. Finally, the exosome pellets were resuspended in 200 μL PBS. The protein content of exosomes was determined by the BCA kit (Beyotime Biotechnology, Shanghai, China).

## Identification of exosomes

The morphology of plasma exosomes was detected by a transmission electron microscope. Briefly, exosomes were fixed with equal volumes of 1% phosphotungstic acid (pH 7.4). A 10 $\mu$ L sample was loaded onto a bronze net with film after washing and holding at room temperature (RT) for 10 min. Then, 10 $\mu$ L of phosphotungstic acid staining solution was added to negatively stain, and it was observed under a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan). A particle and molecular size analyser (Zetasizer Nano ZS; Malvern Instruments) was used to measure the size distribution of the exosomes. The expression of exosomal surface markers, including CD9 (ab92726, abcam, USA), CD63 (ab68418, abcam, USA), and CD81 (ab79559, abcam, USA), were analysed by Western Blot analysis.

### **VSMCs uptake exosomes**

The VSMCs were isolated from 6–8 week-old male C57/BL mice as described before [31]. VSMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco BRL Co. USA), penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. VSMCs were treated with 50 $\mu$ g of ESRD-Ex, RTR-Ex, and Nor-Exo, and the calcification levels were evaluated. Exosomes were labelled with PKH26 (MINI26, Sigma, USA) according to the manufacturer's instructions. Briefly, 4 $\mu$ l of PKH26 fluorescent solution was dissolved in 1ml of diluting solution C, and then the mixed solution was added to 40  $\mu$ l exosomes. After stopping the reaction by using 5% BSA, the mixture was ultracentrifugated at 100,000  $\times$  g for 70 minutes and the labelled exosomes were incubated with VSMCs at 37 °C for 12h. After fixing the cells with 4% paraformaldehyde for 30 minutes at RT and washing the cells 3 times with PBS, DAPI (Invitrogen, Carlsbad, USA) was added for 5 minutes. After washing with PBS 3 times, the staining signals were analysed with a fluorescence microscope (DMI6000B, Leica, Germany).

### **Alizarin Red S staining**

VSMCs were co-incubated with  $\beta$ -glycerophosphate ( $\beta$ -GP) and ESRD-Ex, RTR-Ex or Nor-Ex, respectively, for 21 days, and the Alizarin Red S staining was done as before [31]. The cells were fixed and then stained with 1% (pH 4.2) Alizarin Red S for about 10 minutes. The mineralized nodules were assessed and photographed with a microscope. To quantify calcium levels, the cells were washed with PBS and decalcified with 0.6 N HCl for 24 h. Calcium content was determined by measuring the concentration of calcium in the HCl supernatant by atomic absorption spectroscopy. Following decalcification, the cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The protein content was measured with a BCA protein assay (Beyotime Biotechnology, Shanghai, China). The calcium content of the cell layer was normalized to the protein content.

### **Western Blot analysis**

The expression of proteins was determined by Western Blot as previously described [3]. Briefly, the concentration of protein was detected using a BCA kit (Beyotime, Shanghai, China). 30 $\mu$ g of total protein was loaded onto 8% or 12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were incubated with primary antibody at 4 °C for over 12h after blocking with 5% non-fat milk for 1h.

Subsequently, HRP-conjugated goat-anti-rabbit (sc-2004, 1:5000, Santa Cruz) or HRP-conjugated goat-anti-mouse (sc-2005, 1:5000, Santa Cruz) secondary antibodies were used to incubate with the membrane at RT for 1h. The immunoreactive bands were visualized using the ECL Plus Western Blot Detection Kit (Amersham Biosciences U.K. Ltd). The relative expression levels of proteins were normalized to the intensity of the  $\beta$ -actin band. Primary antibodies including CD9 (ab92726, 1:1,000, Abcam), CD63 (ab68418, 1:1,000, Abcam), CD81 (ab79559, 1:1,000, Abcam), Runx2 (ab76956; 1:1,000, Abcam), Fetuin-A (16571-1-AP, 1:1000, Proteintech), MGP (10734-1-AP, 1:1000, Proteintech), Annexin-A2 (11256-1-AP, 1:1000, Proteintech), BMP-2 (ab214821, 1:1000, Abcam), Rankl (23408-1-AP, 1:500, Proteintech), and  $\beta$ -actin (ab6276, 1:3000, Abcam).

## ELISA analysis

The contents of Fetuin-A (ab108855), MGP (CSB-E09714h), Annexin-A2 (CSB-E12156h), BMP-2 (CSB-E04507h), and Rankl (ab213841) were detected using an ELISA kit according to the manufacturer's instructions. Briefly, 0.1 ml sample or standard were added into each hole of the reaction plate and incubated at 37 °C for 2h. After discarding the liquid, 0.1 ml of biotin labelled antibody working liquid was added and put on the new plate paste, continuing to incubate at 37 °C for another 30 min. Then the liquid was discarded again and washed 3 times. 100 ul working liquid was added, and it was covered with the new post and incubated at 37°C for 1h. The liquid was dropped and washed 5 times. Finally, 90 ul of substrate solution was added to each hole and coloration was done at 37 °C for 15-30 min away from light. Then the OD value (Optical Density) of each hole was measured with a microplate at the wavelength of 450 nm within 5 min after termination of the reaction.

## Statistical analysis

The data are presented as means  $\pm$  standard deviation (SD), and they were analysed with GraphPad Prism software (GraphPad Prism version 6.0). The Student's t-test was used to compare normally distributed data between two different groups, while one-way analysis of variance (ANOVA) together with a Tukey's post hoc test was used for multiple groups. A level of  $p < 0.05$  was considered statistically significant. All experiments were repeated at least three times, and representative images are shown in the figures.

## Abbreviations

ESRD: End-stage renal disease; RTR: renal transplant recipients; Nor: normal; Ex: Exosome; ESRD-Ex: the plasma exosomes derived from ESRD patients; RTR-Ex: the plasma exosomes derived from renal transplant recipients; Nor-Ex: the plasma exosomes derived from normal health control group; VSMCs: vascular smooth muscle cells; DMEM: Dulbecco's Modified Eagle Medium;  $\beta$ -GP: $\beta$ -glycerophosphate; MGP: matrix gla protein; BMP-2: bone morphogenetic protein; Rankl: receptor activator for nuclear factor- $\kappa$  B ligand; CACS: coronary artery calcification scores; TG: triglycerid; TC: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; Cre: creatinine; BUN: blood urea nitrogen; csCa: corrected

serum calcium; P phosphorus; PTH: parathyroid hormone; ucMGP: uncarboxylated MGP; GFR: glomerular filtration rate.

## Declarations

### Ethical approval and consent to participate

The human samples conformed to the principles outlined in the Declaration of Helsinki. All subjects gave their informed consent for inclusion before they participated in the study. All experiments were reviewed and approved by the Ethics Committee of the Second Xiang-Ya Hospital, Central South University.

### Consent for publication

Not applicable.

### Availability of data and materials

All data and materials are available upon request.

### Competing interests

All of authors have no conflict of interest.

### Funding

This work was supported by funding from the National Natural Science Foundation of China (Nos. 81770881 and 81870623).

### Authors' contributions

LQY conceived and designed the experiments. XL and TZ performed the experiments, analyzed the data and prepared all the figures. JYZ, FX, FL, SKS, FW, BG, MHZ, YW, QSX, XBX and XBL provided technical support, made substantial contributions to data analysis and revised the manuscript critically for important intellectual content. XL and TZ wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Acknowledgements

We are grateful to the Department of Kidney Transplantation, affiliated to the Second Xiang-Ya Hospital, Central South University, for providing human arteries.

## References

1. Nitta K, Ogawa T. Vascular calcification in end-stage renal disease patients. *Contrib Nephrol*. 2015;185:156–67.
2. Rangaswami J, Mathew RO, Parasuraman R, Tantisattamo E, Lubetzky M, Rao S, Yaqub MS, Birdwell KA, Bennett W, Dalal P, Kapoor R, Lerma EV, Lerman M, McCormick N, Bangalore S, McCullough P A, and Dadhania D M Cardiovascular disease in the kidney transplant recipient: epidemiology, diagnosis and management strategies. *Nephrol Dial Transplant*, 2019.
3. Lin X, Xu F, Cui RR, Xiong D, Zhong JY, Zhu T, Li F, Wu F, Xie XB, Mao MZ, Liao XB, Yuan LQ. Arterial Calcification Is Regulated Via an miR-204/DNMT3a Regulatory Circuit Both In Vitro and in Female Mice. *Endocrinology*. 2018;159:2905–16.
4. Peng YQ, Xiong D, Lin X, Cui RR, Xu F, Zhong JY, Zhu T, Wu F, Mao MZ. Liao X B, and Yuan L Q Oestrogen Inhibits Arterial Calcification by Promoting Autophagy. *Sci Rep*. 2017;7:3549.
5. Lin X, Zhan JK, Zhong JY, Wang YJ, Wang Y, Li S, He JY, Tan P, Chen YY, Liu XB, Cui XJ, Liu YS. lncRNA-ES3/miR-34c-5p/BMF axis is involved in regulating high-glucose-induced calcification/senescence of VSMCs. *Aging*. 2019;11:523–35.
6. Zhang C, Zhang K, Huang F, Feng W, Chen J, Zhang H, Wang J, Luo P, Huang H. Exosomes, the message transporters in vascular calcification. *J Cell Mol Med*. 2018;22:4024–33.
7. Li FX, Liu JJ, Xu F, Lin X, Zhong JY, Wu F. and Yuan L Q Role of tumor-derived exosomes in bone metastasis. *Oncol Lett*. 2019;18:3935–45.
8. Wu F, Li F, Lin X, Xu F, Cui R, Zhong J, Zhu T, Shan SK, Liao XB, Yuan L, and Mo Z Exosomes increased angiogenesis in papillary thyroid cancer microenvironment. *Endocr Relat Cancer*, 2019.
9. Li S, Zhan JK, Wang YJ, Lin X, Zhong JY, Wang Y, Tan P, He JY, Cui XJ, Chen YY, Huang W, Liu YS. Exosomes from hyperglycemia-stimulated vascular endothelial cells contain versican that regulate calcification/senescence in vascular smooth muscle cells. *Cell Biosci*. 2019;9:1.
10. Lin X, Li S, Wang YJ, Wang Y, Zhong JY, He JY, Cui XJ, Zhan JK, Liu YS Exosomal Notch3 from high glucose-stimulated endothelial cells regulates vascular smooth muscle cells calcification/aging. *Life Sci*, 2019; 232: 116582.
11. Xu F, Zhong JY, Lin X, Shan SK, Bei G, Zheng MH, Wang Y, Li F, Cui RR, Wu F, Zhou E, Liao XB, Liu YS. and Yuan L Q Melatonin alleviates vascular calcification and ageing through exosomal miR-204/miR-211 cluster in a paracrine manner. *J Pineal Res*, 2020; e12631.
12. Leopold JA. Vascular calcification: Mechanisms of vascular smooth muscle cell calcification. *Trends Cardiovasc Med*. 2015;25:267–74.
13. Back M, Aranyi T, Cancela ML, Carracedo M, Conceicao N, Leftheriotis G, Macrae V, Martin L, Nitschke Y, Pasch A, Quaglino D, Rutsch F, Shanahan C, Sorribas V, Szeri F, Valdivielso P, Vanakker O, and Kempf H Endogenous Calcification Inhibitors in the Prevention of Vascular Calcification: A Consensus Statement From the COST Action EuroSoftCalcNet. *Front Cardiovasc Med*. 2018;5:196.
14. Chen NX, Duan D, O'Neill KD, Wolisi GO, Koczman JJ, Laclair R, Moe SM. The mechanisms of uremic serum-induced expression of bone matrix proteins in bovine vascular smooth muscle cells. *Kidney Int*. 2006;70:1046–53.

15. Davenport C, Harper E, Forde H, Rochfort KD, Murphy RP, Smith D, Cummins. P M RANKL promotes osteoblastic activity in vascular smooth muscle cells by upregulating endothelial BMP-2 release. *Int J Biochem Cell Biol.* 2016;77:171–80.
16. Chen NX, O'Neill KD, Chen X, Moe SM. Annexin-mediated matrix vesicle calcification in vascular smooth muscle cells. *J Bone Miner Res.* 2008;23:1798–805.
17. Kapustin AN, Davies JD, Reynolds JL, McNair R, Jones GT, Sidibe A, Schurgers LJ, Skepper JN, Proudfoot D, Mayr M, Shanahan CM. Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ Res.* 2011;109:e1–12.
18. Xu F, Li FX, Lin X, Zhong JY, Wu F, Shan SK, Tan CM, Yuan LQ., and Liao X B Adipose tissue-derived omentin-1 attenuates arterial calcification via AMPK/Akt signaling pathway. *Aging.* 2019;11:8760–76.
19. Viegas CSB, Santos L, Macedo AL, Matos AA, Silva AP, Neves PL, Staes A, Gevaert K, Morais R, Vermeer C, Schurgers L, Simes DC. Chronic Kidney Disease Circulating Calciprotein Particles and Extracellular Vesicles Promote Vascular Calcification: A Role for GRP (Gla-Rich Protein). *Arterioscler Thromb Vasc Biol.* 2018;38:575–87.
20. Metzger M, Houillier P, Gauci C, Haymann JP, Flamant M, Thervet E, Boffa JJ, Vrtovsnik F, Froissart M, Stengel B. and Urena-Torres P Relation between circulating levels of 25(OH) vitamin D and parathyroid hormone in chronic kidney disease: quest for a threshold. *J Clin Endocrinol Metab.* 2013;98:2922–8.
21. Viegas CS, Rafael MS, Enriquez JL, Teixeira A, Vitorino R, Luis IM, Costa RM, Santos S, Cavaco S, Neves J, Macedo AL, Willems BA, Vermeer C. and Simes D C Gla-rich protein acts as a calcification inhibitor in the human cardiovascular system. *Arterioscler Thromb Vasc Biol.* 2015;35:399–408.
22. Marechal C, Schlieper G, Nguyen P, Kruger T, Coche E, Robert A, Floege J, Goffin E, Jadoul M. and Devuyst O Serum fetuin-A levels are associated with vascular calcifications and predict cardiovascular events in renal transplant recipients. *Clin J Am Soc Nephrol.* 2011;6:974–85.
23. Schurgers LJ, Uitto J, Reutelingsperger CP, Vitamin. K-dependent carboxylation of matrix Gla-protein: a crucial switch to control ectopic mineralization. *Trends Mol Med.* 2013;19:217–26.
24. Thamratnopkoon S, Susantitaphong P, Tumkosit M, Katavetin P, Tiranathanagul K, Praditpornsilpa K, Eiam-Ong S. Correlations of Plasma Desphosphorylated Uncarboxylated Matrix Gla Protein with Vascular Calcification and Vascular Stiffness in Chronic Kidney Disease. *Nephron.* 2017;135:167–72.
25. Nollet L, Van Gils M, Verschuere S, Vanakker O. The Role of Vitamin K and Its Related Compounds in Mendelian and Acquired Ectopic Mineralization Disorders. *Int J Mol Sci,* 2019; 20.
26. Holt SG, Smith ER. Fetuin-A-containing calciprotein particles in mineral trafficking and vascular disease. *Nephrol Dial Transplant.* 2016;31:1583–7.
27. Scialla JJ, Kao WH, Crainiceanu C, Sozio SM, Oberai PC, Shafi T, Coresh J, Powe NR, Plantinga LC. Jaar B G, and Parekh R S Biomarkers of vascular calcification and mortality in patients with ESRD. *Clin J Am Soc Nephrol.* 2014;9:745–55.

28. New SE, Goettsch C, Aikawa M, Marchini JF, Shibasaki M, Yabusaki K, Libby P, Shanahan CM, Croce K, Aikawa E. Macrophage-derived matrix vesicles: an alternative novel mechanism for microcalcification in atherosclerotic plaques. *Circ Res.* 2013;113:72–7.
29. Chen J, Budoff MJ, Reilly MP, Yang W, Rosas SE, Rahman M, Zhang X, Roy JA, Lustigova E, Nessel L, Ford V, Raj D, Porter AC, Soliman EZ, Wright JT Jr. Wolf M, and He J Coronary Artery Calcification and Risk of Cardiovascular Disease and Death Among Patients With Chronic Kidney Disease. *JAMA Cardiol.* 2017;2:635–43.
30. Bashir A, Moody WE, Edwards NC, Ferro CJ, Townend JN, Steeds RP. Coronary Artery Calcium Assessment in CKD: Utility in Cardiovascular Disease Risk Assessment and Treatment? *Am J Kidney Dis.* 2015;65:937–48.
31. Lin X, Li F, Xu F, Cui RR, Xiong D, Zhong JY, Zhu T, Shan SK, Wu F, Xie XB. Liao X B, and Yuan L Q Aberration methylation of miR-34b was involved in regulating vascular calcification by targeting Notch1. *Aging.* 2019;11:3182–97.

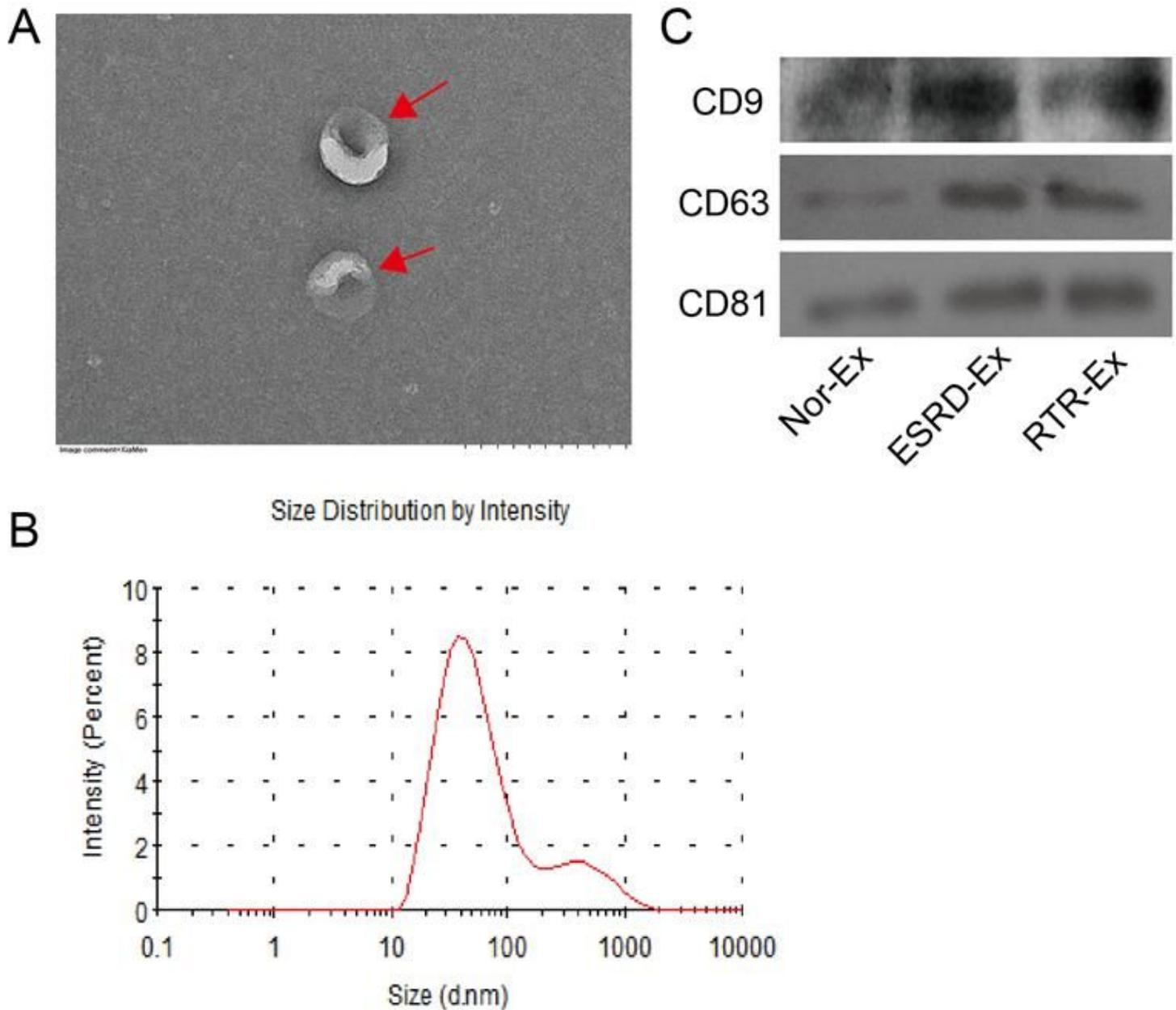
## Tables

**Table 1. Biochemical characteristics in Normal health, ESRD and RTR**

	Normal	ESRD	RTR
Age (years)	37.75 ± 4.333	33.13 ± 1.42	39.88 ± 2.844
Cre (umol/l)	61.56 ± 3.327	1284 ± 143.2	117.1 ± 15.99*#
BUN (mmol/l)	4.763 ± 0.5495	29.4 ± 3.669*	7.598 ± 0.9367*#
UA (umol/l)	307.7 ± 20.34	438.1 ± 25.65*	375.1 ± 32.18*#
csCa (mg/dl)	8.753 ± 0.1139	9.575 ± 0.3389*	9.176 ± 0.1603
P (mg/dl)	3.042 ± 0.1964	6.312 ± 0.8433*	2.767 ± 0.2039#
Ca×P (mg <sup>2</sup> /d1 <sup>2</sup> )	26.58 ± 1.624	59.95 ± 7.89*	25.49 ± 2.1#
PTH (mmol/l)	4.999 ± 0.4806	61.26 ± 10.89*	9.089 ± 1.015#
TG (mmol/l)	1.474 ± 0.2042	2.368 ± 0.6717	1.425 ± 0.1632
TC (mmol/l)	3.363 ± 0.275	4.276 ± 0.4811	4.12 ± 0.3546
HDL (mmol/l)	0.905 ± 0.08356	1.075 ± 0.0964	1.289 ± 0.1698
LDL (mmol/l)	2.099 ± 0.2188	2.581 ± 0.4126	2.393 ± 0.1939
25(OH)D (nmol/l)	50.63 ± 5.018	35.5 ± 3.157*	40.88 ± 3.108

**Abbreviation:** Cre: creatinine; BUN: blood urea nitrogen; UA: uric acid; csCa: corrected serum calcium; P: phosphorus; PTH: parathyroid hormone; TG: triglyceride; TC: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein. \* $p < 0.05$ , compared with Normal group. # $p < 0.05$ , compared with ESRD group.

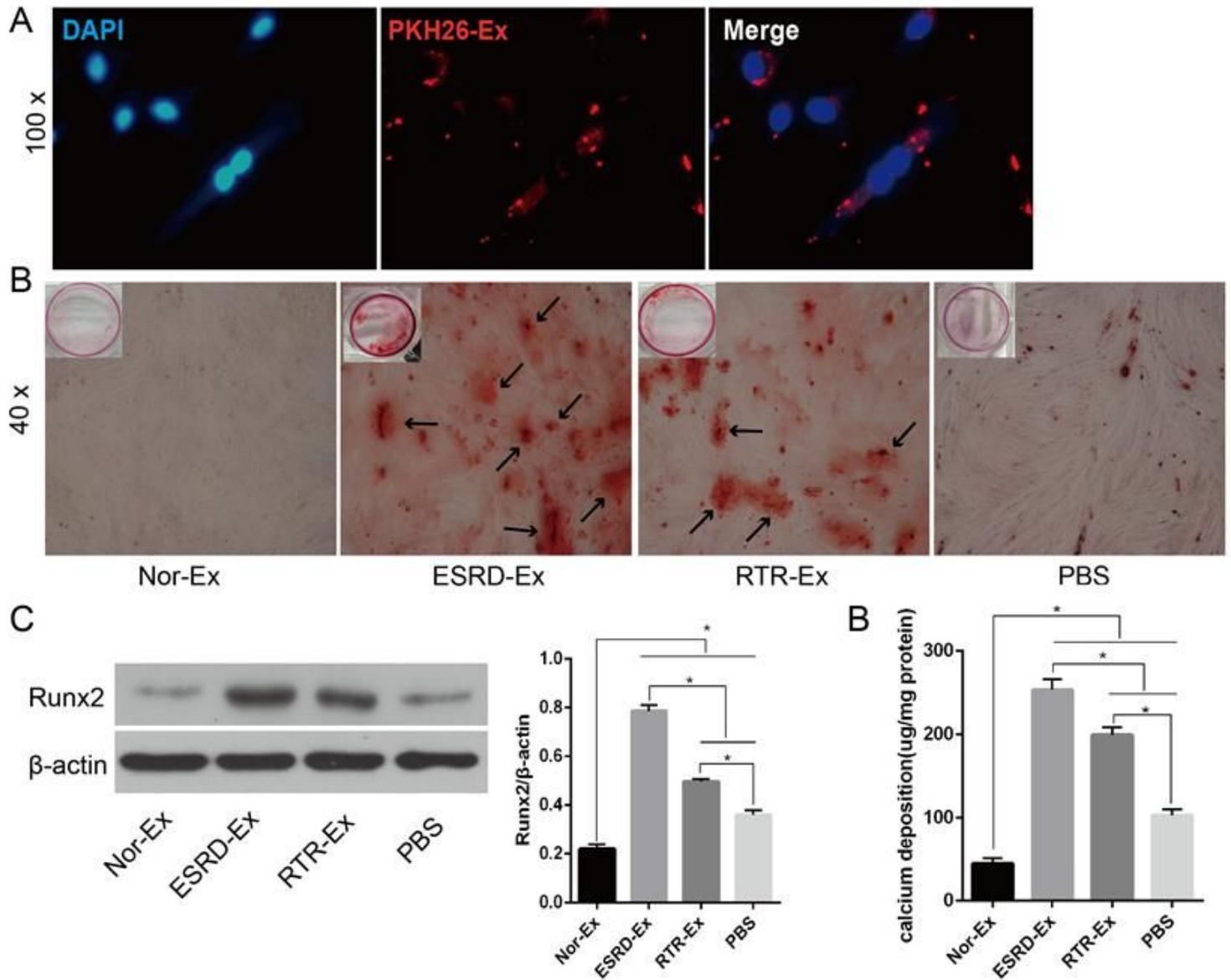
## Figures



**Figure 1**

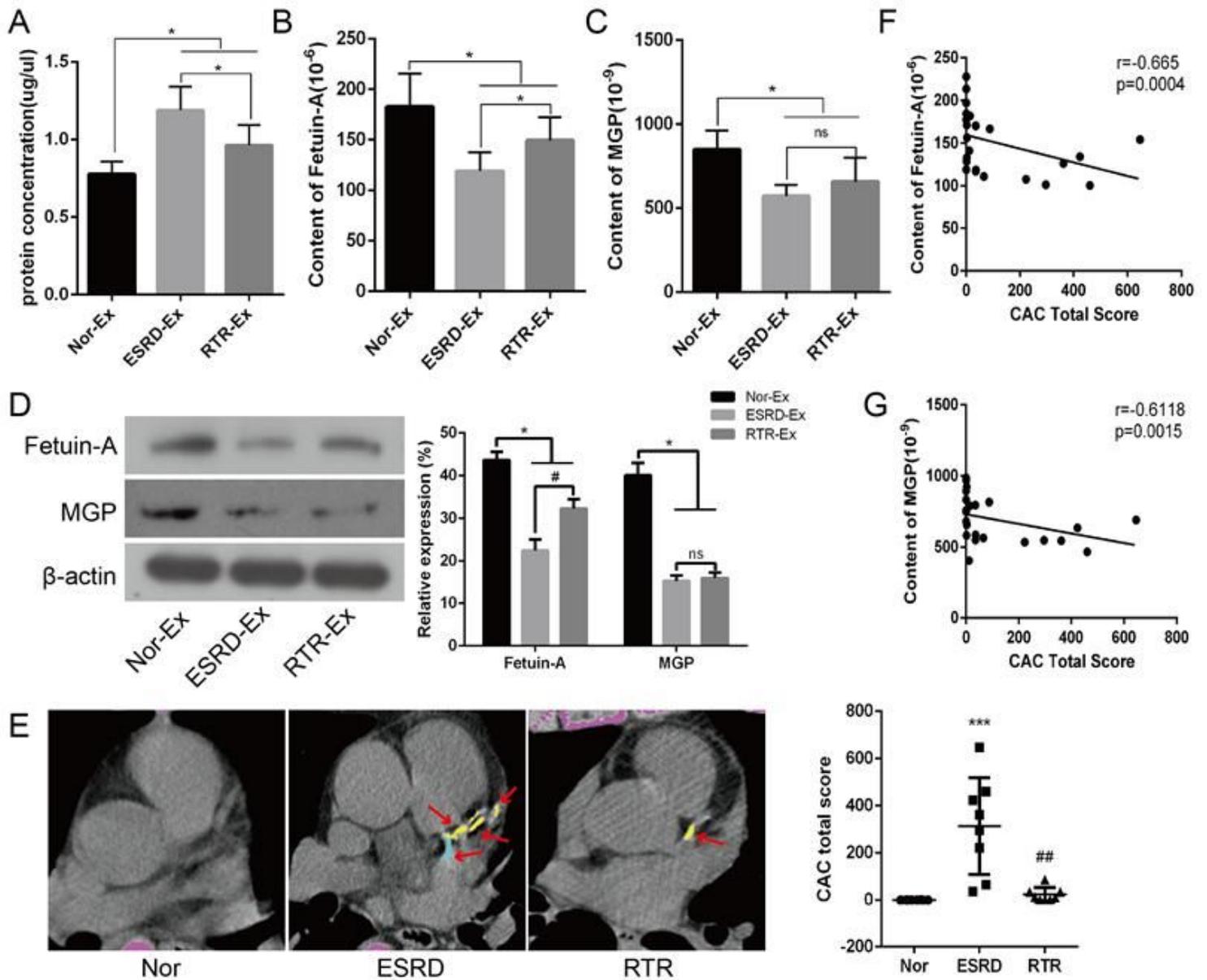
The characteristics of exosomes. (A) The morphology of exosomes was observed by transmission electron microscopy. The scale bar was 500nm. n=3. (B) The diameter distribution of exosomes was

measured by a molecular size analyser. (C) Exosome markers CD9, CD63, and CD81 were detected by Western Blot. The images are shown.



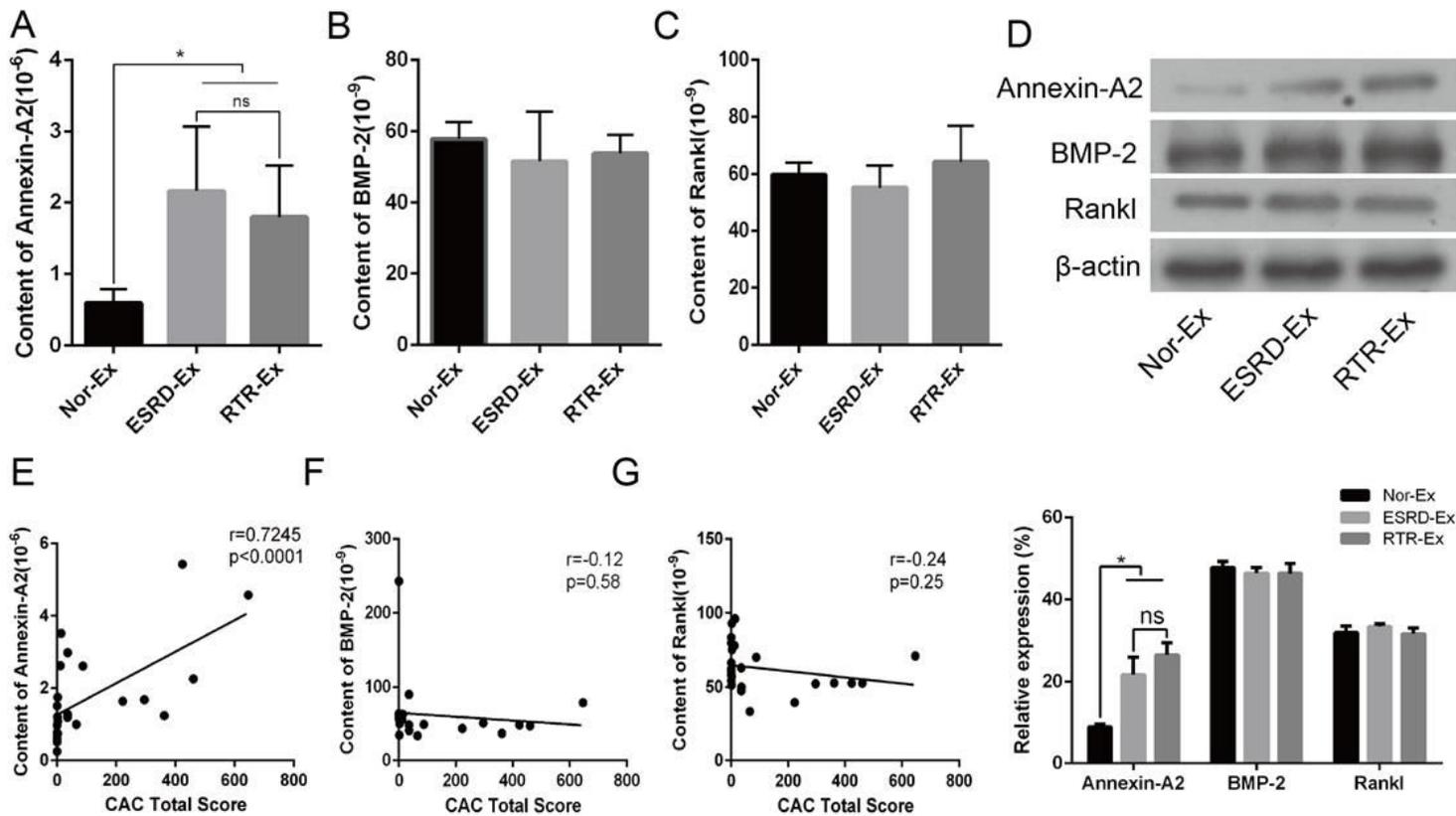
**Figure 2**

ESRD-Ex could be uptaken by VSMCs and promote VSMCs calcification. (A) The CACS of normal health, ESRD, and RTR were calculated by a multi-layer spiral computed tomographic. (B) Fluorescence microscopy analysis revealed that PKH26-labeled-Ex could be uptaken and incorporated into VSMCs. (C) Alizarin Red S staining showed the mineralized nodules in VSMCs, and the calcium contents were extracted with cetyl-pyridinium chloride and quantified by spectrophotometry. (D) The expression of Runx2 was measured by Western Blot in VSMCs treated with different kinds of exosomes. The presentative images are shown.  $n=3$ ,  $*p<0.05$ . CACS: coronary artery calcification score.



**Figure 3**

Fetuin-A and MGP were decreased in ESRD-Ex. (A) The different protein concentration of exosomes in Nor-Ex, ESRD-Ex and RTR-Ex. (B and C) The content of Fetuin-A and MGP in Nor-Ex, ESRD-Ex, and RTR-Ex were measured by ELISA. (D) Western Blot analysis detected the protein levels of Fetuin-A and MGP.  $n=3$ ,  $*p<0.05$ . (E and F) The Spearman analysis showed the correlations between the plasma content of Fetuin-A and MGP with CACS, respectively.  $n=24$ . ns: not significant.



**Figure 4**

Annexin-A2 increased in ESRD-Ex. (A-C) The content of Annexin-A2, BMP-2 and Rankl in Nor-Ex, ESRD-Ex and RTR-Ex were measured by ELISA. (D) Western Blot detected the protein levels of Annexin-A2, BMP-2, and Rankl.  $n=3$ ,  $*p<0.05$ . (E-G) The Spearman analysis showed the correlations between the plasma content of Annexin-A2, BMP-2, and Rankl with CACS, respectively.  $n=24$ . ns: not significant.