

## **Polysaccharide-Protein Complex-Decorated Selenium Nanosystem as Efficient Bone-Formation Therapeutics**

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### **Experimental section**

#### **Preparation of PTR-SeNPs**

In brief, 4 mL of aqueous polysaccharide-protein complex (PSP, 1.5mg/mL) was mixed with 1 mL of aqueous sodium selenite (25 mM) under magnetic stirring for 2 hours before drop-wise addition of 1 mL of freshly prepared ascorbic acid (100 mM). After reconstituting with ultrapure water to 10mL, the mixture was stirred for 10 min under room temperature and allowed to react for 24 hours at 4°C before extensive dialysis (*Mw* cutoff: 8,000) until the total dissolved solute (TDS) was similar to that of Milli-Q water. The resulting dialysate, which is the PTR-SeNPs was stored at 4°C prior to further composition analysis and structure characterization.

#### **Characterization**

The concentration of Se in PTR-SeNPs was determined using Agilent 710 ICP-OES (Agilent Technologies Inc., USA) using 196.026 as emission wavelength and 203.985

as reference. The particle size of specimen was then visualized by JEM-2100F Field Emission Electron Microscope (JEOL Ltd., Japan) with magnification of 20,000 $\times$ , 40,000 $\times$  and 150,000 $\times$  using 200kV electrons beam. Single nanoparticle was selected under magnification of 150,000 $\times$  and its elemental composition and crystal structure were further investigated by using HR-TEM-EDX (JEOL JEM-2100F + Horiba EX-250). All images obtained were analyzed by software ImageJ. Stability of PTR-SeNPs was measured by Nanosight NS300 (Malvern Instruments Ltd., UK) using nanoparticles tracking analysis (NTA). FTIR spectra were determined and compared by using Nicolet™ iSTM 50 Spectrometer.

### **Cell culture**

MC3T3-E1 subclone 4 preosteoblast cells were purchased from American Type Culture Collection (ATCC ® CRL-2593, Rockville, MD, USA) and maintained in ascorbic acid free  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM; Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA, USA), 1% 100 $\times$  antibiotics and anti-mycotics (Invitrogen, San Diego, CA, USA) in a 37°C incubator with 95% relative humidity and 5% CO<sub>2</sub>. Cells were routinely passaged using 0.25% trypsin/0.1% EDTA at 70 – 90% confluence.

### **Cellular uptake behavior of PTR-SeNPs**

In brief, MC3T3-E1 cells were firstly seeded in a 35 mm confocal dish for 24 hours followed by sequential staining with Hoechst 33342 (1 $\mu$ g/mL; nucleus; Invitrogen, CA, USA) and LysoTracker Deep Red (75 $\mu$ M; lysosomes; Invitrogen, CA, USA) for 20 and 30min, respectively. After further incubation with 10 $\mu$ M of coumarin-6-loaded PTR-SeNPs at 37°C for 10 min, the cellular uptake behavior of PTR-SeNPs by MC3T3-E1 cells was intracellularly traced by fluorescence microscopy.

### ***In vitro* osteogenic effect of PTR-SeNPs**

#### **Cell proliferation assay**

In brief, MC3T3-E1 cells were firstly seeded into 96-well tissue culture plates at 15,000 cells/well. Cells pretreated with different concentrations of PTR-SeNPs (10nM - 10 $\mu$ M), 6 $\mu$ g/mL PTR-PSP or 10 $\mu$ M sodium selenite were then incubated for 24, 48 and 72 hour followed by determining cell proliferation using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

assay (Promega). To perform MTS assay, 4.6 mg of phenazine methosulfate (PMS) was dissolved in 5mL of PBS, while 42mg of MTS powder was dissolved in 21mL of PBS with pH adjustment to 6.0 – 6.5. Subsequently, 4.2 mL of MTS solution was mixed with 1mL of PMS solution. After filtered with 0.22 $\mu$ M filter, cells were added into the MTS/PMS mixture (20 $\mu$ L/well) and incubated for 1-2 hours at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Absorbance was determined at 490nm using a microplate reader (Clariostar, USA).

### **Osteoblastic differentiation**

MC3T3-E1 preosteoblast cells cultured in osteogenic medium containing 10nM  $\beta$ -glycerophosphate and 50  $\mu$ g/mL ascorbic acid were pretreated with 10 $\mu$ M of PTR-SeNPs for 7 days. ALP activity in the MC3T3-E1 cells was then determined by spectrophotometry at 410 nm, while ALP-containing cells (osteoblasts) were visualized by ALP staining kit. Fresh culture media were replenished every 3 – 4 days.

### **ALP activity**

In brief, MC3T3-E1 cells pretreated with 10 $\mu$ M of PTR-SeNPs for 7 days were washed with PBS followed by lysed with 50mM Tris-HCl (200 $\mu$ L/well, pH 8.0) and 0.5% Triton X-100. Cell lysates were then harvested and transferred into 1.5mL centrifuge tubes before storage at -80°C. After defrosted, all lysates were centrifuged (21,000  $\times$  g; 20 min; 4°C) and 10 $\mu$ L of supernatant was added into 96 wells in triplicate followed by incubation with 200 $\mu$ L of 10mM *p*-nitrophenyl phosphate (*p*NPP) in 1M diethanolamine and 0.5mM MgCl<sub>2</sub> (pH 9.8) for 15-30 min. Consequently, the reaction was quenched by adding 50 $\mu$ L of 3M NaOH and the resulting absorbance was measured at 410nm using a microplate reader. To normalize the ALP activity, the total protein content was also determined by BCA assay. In short, 200 $\mu$ L of BCA solution (mixing BCA reagent A with B as indicated in manufacture's manual) was added into a well containing 2 $\mu$ L of protein sample and 23 $\mu$ L of water followed by incubation at 37°C for 30 min. Similarly, the resulting absorbance was measured at 595nm using a microplate reader.

### **ALP staining**

In brief, MC3T3-E1 cells pretreated with 10 $\mu$ M of PTR-SeNPs for 7 days were washed with PBS and fixed with 10% formalin for 15 min. The ALP in cells were

then stained by Fast Blue RR salt and Naphthol AS-MX phosphate alkaline solution for 60 min followed by rinsing thoroughly with deionized water.

### **Mineralization assay**

MC3T3-E1 cells cultured in osteogenic medium containing 10nM  $\beta$ -glycerophosphate and 50  $\mu\text{g}/\text{mL}$  ascorbic acid were pretreated with different concentration of PTR-SeNPs (10 $\mu\text{M}$ ) for 16 days. Bone nodules formed in the MC3T3-E1 cells were then visualized and compared by Von Kossa and Alizarin Red S staining.

### **Von Kossa staining**

To detect calcium deposit, cells were pre-washed with PBS and fixed with 10% formalin for 15 min. Fixed cells were then treated with 5% silver nitrate for 30 min under light following by sequential washing with deionized water, 5% sodium carbonate in 10% formalin (1 min), deionized water and 5% sodium thiosulfate (5 min). Consequently, stained well was captured and analyzed by ImageJ to quantify calcium deposition.

### **Alizarin Red S staining**

Similarly, cells were pre-washed with PBS and fixed with 10% formalin for 15 min. Fixed cells were then stained with 40nM Alizarin Red S solution (pH 4.1) for 20 min with gently shaking followed by heavy washing 4 times with 4mL of deionized water for 5 min per well. Excessive water in the wells was removed and the resulting image was captured. To quantify the calcium deposit, 800 $\mu\text{L}$  of 10% (v/v) acetic acid was added into each well followed by incubation with shaking for further 30 min. All stained cells were then scraped with cell scraper and transferred into a 1.5mL centrifuge tube using acetic acid. After vortex-mixing for 30 sec, 500 $\mu\text{L}$  of mineral oil was added and the slurry was heated up to 85°C for 10 min followed by cooling down in ice for 5 min. All tubes were then centrifuged at 20,000  $\times$  g for 15 min and 500 $\mu\text{L}$  of the supernatant was transferred into new Eppendorf tubes. After adding 200 $\mu\text{L}$  of 10% (v/v) ammonium hydroxide, the mixture was aliquot in a 96 well plate in triplicate followed by measuring the absorbance with a microplate reader at 405nm.

### **Total RNA isolation**

mRNA expression of major osteogenic markers (such as ALP, Dlx5, Runx2, Osx, OCN and OPG/RANKL) in the MC3T3-E1 cells pretreated with 10 $\mu\text{M}$  of PTR-

SeNPs for 3, 7 and 16 days were quantified by real-time PCR. For total RNA extraction, cells were washed twice with 1mL of ice cold PBS and total RNA was isolated with 1mL of Trizol reagent per 6 wells according to manufacturer's protocol (Life technologies, USA). After homogenization, 200 $\mu$ L of chloroform was added with gentle shaking and the mixture was allowed to stand for 3 min at room temperature. All samples were then centrifuged (12,000  $\times$  g; 10 min; 4 $^{\circ}$ C) and the top aqueous layers were transferred into new tubes prior to adding 500 $\mu$ L of 2-propanol. Subsequently, the whole reaction mixture was centrifuged under the same conditions and the 2-propanol was removed followed by washing with 1mL of 75% ethanol in DEPC H<sub>2</sub>O. After centrifugation (7,500  $\times$  g; 5 min; 4 $^{\circ}$ C), the 75% ethanol was removed and the RNA pellet was air-dried. Once the RNA pellets turned into milky, 10 – 20  $\mu$ L of DEPC H<sub>2</sub>O was added to re-dissolve the RNA and stored at -80 $^{\circ}$ C until further qPCR analysis.

#### **Real-time polymerase chain reaction (qPCR)**

In brief, 20 ng of RNA was firstly prepared by One-Step SYBR Green RT-qPCR according to manufacturer's instructions. In a 20  $\mu$ L reaction, the reaction mixture contained 10  $\mu$ L of 2X One Step SYBR RT-PCR Buffer 4, 0.8 $\mu$ L of PrimeScript One-Step Enzyme Mix 2, 0.4  $\mu$ M of Forward PCR primers, 0.4 $\mu$ M of Reverse PCR primers and 20ng of RNA sample. qPCR analysis of BMP-2 and major osteogenic markers (such as ALP, DLx5, Runx2, Osx, OCN and OPG/RANKL) was performed by a Pikoreal thermal cycler system (Thermo, USA) using thermal cycling program and primers shown in Table 2 and 3, respectively. Relative mRNA expression of each osteogenic marker gene was normalized to that of GAPDH.

**Table 1.** Thermal cycling program of qPCR analysis (mouse)

| Stage                              | Temperature     | Hold Time |
|------------------------------------|-----------------|-----------|
| Reverse transcription<br>(1 cycle) | 42 $^{\circ}$ C | 5 min     |
|                                    | 95 $^{\circ}$ C | 10 sec    |
| PCR reaction<br>(40 cycle)         | 95 $^{\circ}$ C | 5 sec     |
|                                    | 60 $^{\circ}$ C | 20 sec    |
| Melting curve analysis             | 95 $^{\circ}$ C | 0 sec     |
|                                    | 65 $^{\circ}$ C | 15 sec    |
|                                    | 95 $^{\circ}$ C | 0 sec     |

**Table 2.** Primer list for qPCR analysis (mouse)

| <b>Gene</b> | <b>Forward Primer (5' – 3')</b> | <b>Backward Primer (5' – 3')</b> |
|-------------|---------------------------------|----------------------------------|
| Runx2       | AAGTGCGGTGCAAACCTTTTCT          | TCTCGGTGGCTGGTAGTGA              |
| ALP         | AACCCAGACACAAGCATTCC            | GAGAGCGAAGGGTCAGTCAG             |
| Dlx5        | GTTTCAGAAGACTCAGTACCT           | TGACTGTGGCGAGTTACAC              |
| BMP2        | CCCCAAGACACAGTTCCTA             | GAGACCGCAGTCCGTCTAAG             |
| Osx         | AGAGGTTCACTCGCTCTGACGA          | TTGCTCAAGTGGTCGCTTCTG            |
| OCN         | TAGTGAACAGACTCCGGCGCTA          | TGTAGGCGGTCTTCAAGCCAT            |
| OPG         | ACGAACTGCAGCACATTTGG            | TGTTTCCGGAACACACGTTG             |
| RANKL       | ACGCCAACATTTGCTTTCGG            | ATTGCCCGACCAGTTTTTCG             |
| GAPDH       | CATGGCCTTCCGTGTTCTA             | CCTGCTTACCACCTTCTTGAT            |

### **Total protein extraction**

With a purpose to dissect the BMP-2 signaling (both Smad dependent and independent pathways) mediated by PTR-SeNPs during osteoblast differentiation, protein expression and phosphorylation of Smad1/5/8 ERK, p38 and JNK in the MC3T3-E1 cells pretreated with 10 $\mu$ M of PTR-SeNPs for 4 days were determined by Western blot analysis. For total protein extraction, cells were washed twice with PBS and lysed with 50 $\mu$ L of RIPA Lysis buffer containing PMSF and 1X protease and phosphatase inhibitor cocktail. Cells were then scraped and transferred into 1.5mL centrifuge tubes followed by sonication (1 min) and centrifugation (22,000  $\times$  g; 20 min; 4 $^{\circ}$ C). Protein content of the supernatant was determined by BCA protein assay. After mixed with 5X sample buffer, the reaction mixture was heated up to 95 $^{\circ}$ C for 5 min and stored at -20 $^{\circ}$ C prior to Western blot analysis.

### **Western blot analysis**

For Western blotting, 40 $\mu$ g of protein sample was loaded and separated by 10% SDS-PAGE using gel electrophoresis. Separated proteins were then transferred to PVDF

membranes using iBlot 2 Dry Blotting System. After blocking with 5% BSA (w/v) or 5% blocking grade non-fat dry milk powder (Bio-rad, USA) in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 hour at room temperature, all membranes were incubated with specific primary antibodies including p-Smad 1/5/8, Smad 1/5/8, p-ERK, ERK, p-p38, p-38, p-JNK, JNK and GAPDH [dilution: 1:1000; 5% BSA in TBST] at 4°C overnight. Subsequently, all membranes were extensively washed with TBST and incubated with the secondary anti-mouse IgG HRP-linked antibodies at 1:2000 dilution for 1 hour at room temperature. Finally, the membranes were further washed with TBST, while chemiluminescence for detection was developed using EMD Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore, USA) according to the manufacturer's instructions. Image acquisition was performed using Azure<sup>TM</sup> c600 digital imager (Azure Biosystem Inc., USA) followed by quantification with ImageJ.

### **Japanese Medaka**

Orange-red, outbred line of Japanese medaka (*Oryzia latipes*) was originated from the Molecular Aquatic Toxicology Laboratory of Duke University. A brood colony was maintained at 100-200 individuals (60-80 fish × 3 tanks) at any given time. This brood colony produced all the embryos for the project. In brief, adult fish in brood colony was maintained at 25°C at 14:10 light/dark cycle to provide favorable conditions for egg laying. Under this condition medaka fish produce eggs daily. Fifty percent of tank water was changed daily with de-chlorinated tap water and was maintained at pH 8.0 and 30‰ salinity. Water pH and temperature was monitored daily; nitrate, nitrite, ammonia and water hardness were checked every month. Fish were fed twice daily with commercial fish feed, Otohime β1 (Nisshin Co, Japan), and supplemented with hatched brine shrimp *Artemia nauplii* (Lucky Brand, O.S.I., USA) for 3 days per week. Embryo less than 4 hours post-fertilization was collected and transferred to a petri dishes containing de-chlorinated water for culturing.

### **Japanese medaka embryo culture**

All collected medaka embryos were firstly cultured in a petri dish using saturated sea salt water for 1 day-disinfection followed by culturing in de-chlorinated water. Embryos were cleaned and dead eggs were pick out every day during change of water in order to prevent contamination. Embryos were cultured at 25 – 30°C until hatched

and larvae were transferred to new containers before performing PTR-SeNPs exposure experiment.

### **Fish diet preparation**

In brief, 10 g of commercial fish feed (Otohime  $\beta$ 1) was firstly mixed with 10mL of PTR-SeNPs to make a PTR-SeNPs based diet containing 10 ppm Se. After freeze-drying, the PTR-SeNPs based diet was grinded by using mortar and pestle to pass through a 100 $\mu$ m sieve. For control diet, 10mL of MilliQ water was used instead. Final Se concentration of both control and SeNP-based diets were measured by ICP-MS before use.

### **PTR-SeNPs exposure experiment using medaka larvae**

After collecting the 5 days post-hatched medaka larva, both control and PTR-SeNPs treatment groups were cultured in 6-well cell-culture plates (one medaka larvae per well) and fed the control or PTR-SeNPs based diet (10ppm) twice daily for a total of 11 days. During fish sampling, medaka larvae were anaesthetized by immersing in ice-cold aquarium water for 30 sec.

Similar to the *in vitro* study, the mRNA expression of major osteogenic markers (such as ALP, Runx2, Osx and OPG/RANKL) in the medaka larvae fed with 10ppm of PTR-SeNPs for 2 days were quantified by qPCR. As mentioned in 2.7.4 and 2.7.5, total RNA isolation from medaka larvae (n=3) were performed using Trizol reagent followed by One-Step SYBR Green RT-qPCR analysis using Pikoreal thermal cycler system (Thermo, USA). Thermal cycling program and primers used are shown in Table 1 and Table 3, respectively. Relative mRNA expression of each osteogenic marker gene was normalized to that of 18S.

### **Bone mineralization of medaka larvae**

Mineralization is the end point of bone formation. Bone and cartilage in medaka larvae after treatment with 10ppm of PTR-SeNPs for 11 days (n = 6) were double-stained by Alizarin Red S and Alcian Blue. In brief, medaka larvae were firstly fixed in 10% formalin (2 hours) followed by dehydration using 50% ethanol (10 min). After washing three times with PBS (10 min), medaka larvae were stained overnight with 0.02% Alcian blue 8GX in 70% ethanol containing 0.06M MgCl<sub>2</sub>. Subsequently, medaka larvae were washed three times with absolute ethanol (10 min) followed by sequential washing with 90%, 75% 50%, 25% ethanol and finally PBS (20 min each).



After bleaching with 1.5% H<sub>2</sub>O<sub>2</sub> in 1% KOH (40 min) and washing three times with PBS (10 min), medaka larvae were further digested with 1% trypsin solution in 30% saturated sodium borate solution (40 min). For bone staining, digested larvae were washed three times with PBS (10 min) and stained with saturated Alizarin Red S in 0.5% KOH overnight followed by de-staining three times with 0.5% KOH (10 min). After sequential washing with 20% glycerol/0.25% KOH and 50% glycerol/0.25% KOH, all medaka larvae specimens were stored in 100% glycerol at 4°C prior to examination using stereomicroscope Nikon SMZ18 and upright microscope Eclipse Ci-E.

### **Total RNA isolation**

To further characterize the direct effects of PTR-SeNPs on osteogenesis *in vivo*, mRNA expression of major osteogenic markers (such as ALP, Runx2, Osx and OPG/RANKL) in medaka larvae pretreated with 10ppm of PTR-SeNPs for 2 days were quantified by real-time PCR. For total RNA extraction, total RNA of 3 larvae were isolated with 1mL of Trizol reagent according to manufacturer's protocol (Life technologies, USA). After homogenization, 200µL of chloroform was added with gentle shaking and the mixture was allowed to stand for 3 min at room temperature. All samples were then centrifuged (12,000 × g; 10 min; 4°C) and the top aqueous layers were transferred into new tubes prior to adding 500µL of 2-propanol. Subsequently, the whole reaction mixture was centrifuged under the same conditions and the 2-propanol was removed followed by washing with 1mL of 75% ethanol in DEPC H<sub>2</sub>O. After centrifugation (7,500 × g; 5 min; 4°C), the 75% ethanol was removed and the RNA pellet was air-dried. Once the RNA pellets turned into milky, 10 - 20µL of DEPC H<sub>2</sub>O was added to re-dissolve the RNA and stored at -80°C until further qPCR analysis.

### **Real-time polymerase chain reaction (qPCR)**

In brief, 50ng of RNA was firstly prepared by One-Step SYBR Green RT-qPCR according to manufacturer's instructions. In a 20µL reaction, the reaction mixture contained 10µL of 2X One Step SYBR RT-PCR Buffer 4, 0.8µL of PrimeScript One-Step Enzyme Mix 2, 0.4µM of Forward PCR primers, 0.4µM of Reverse PCR primers and 20ng of RNA sample. All qPCR analysis of those major osteogenic markers (such as ALP, Runx2, Osx and OPG/RANKL) was performed by a Pikoreal thermal cycler

system (Thermo, USA) using thermal cycling program and primers shown in Table 1 and 3, respectively. Relative mRNA expression of each osteogenic marker gene was normalized to that of 18S.

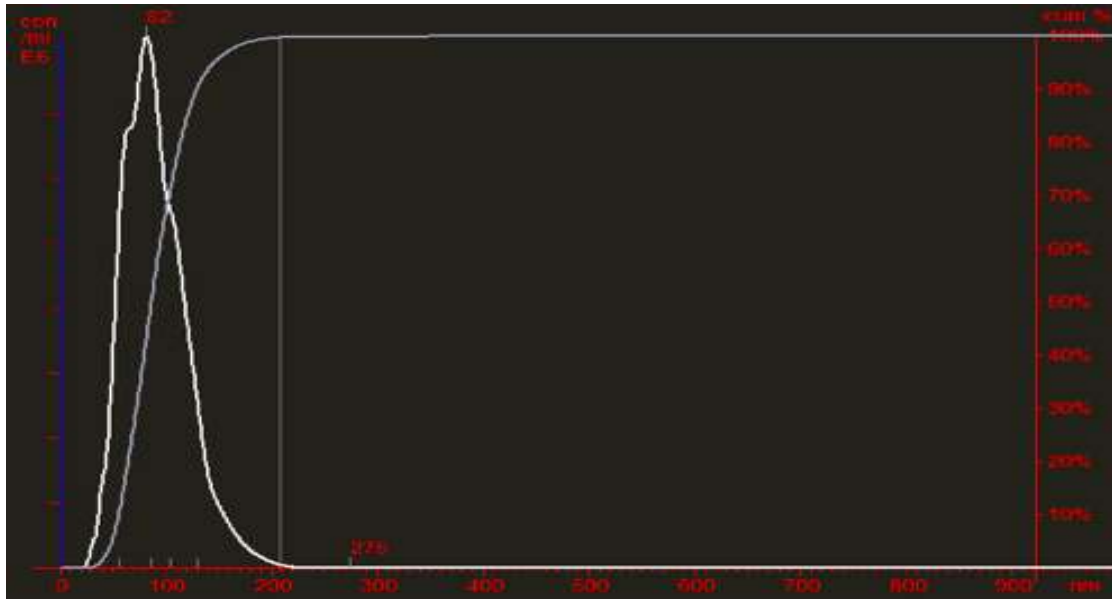
**Table 3.** Primer list for qPCR analysis (medaka larvae)

| <b>Gene</b> | <b>Forward Primer (5' – 3')</b> | <b>Backward Primer (5' – 3')</b> |
|-------------|---------------------------------|----------------------------------|
| Runx2       | TCAGACGCAGTCACAGA               | ACTCAGGTAGGACGGATA               |
| ALP         | AGGGAAGCCGATGTTTCCTG            | TCCTGCATCTTTGGCCCATT             |
| Osx         | TCTCCCCTCAGCTTCCTTAG            | CTGTGTTGCAGACAGCCAGT             |
| OPG         | GCTGGTTATGTGCGTAAA              | AAGAATGCTGAGAGGAA                |
| RANKL       | CTGCTCCGCCACTACAA               | GGGTTGCCAATATCCACAG              |
| 18S         | CATGGCCTTCCGTGTTTCCTA           | CCTGCTTCACCACCTTCTTGAT           |

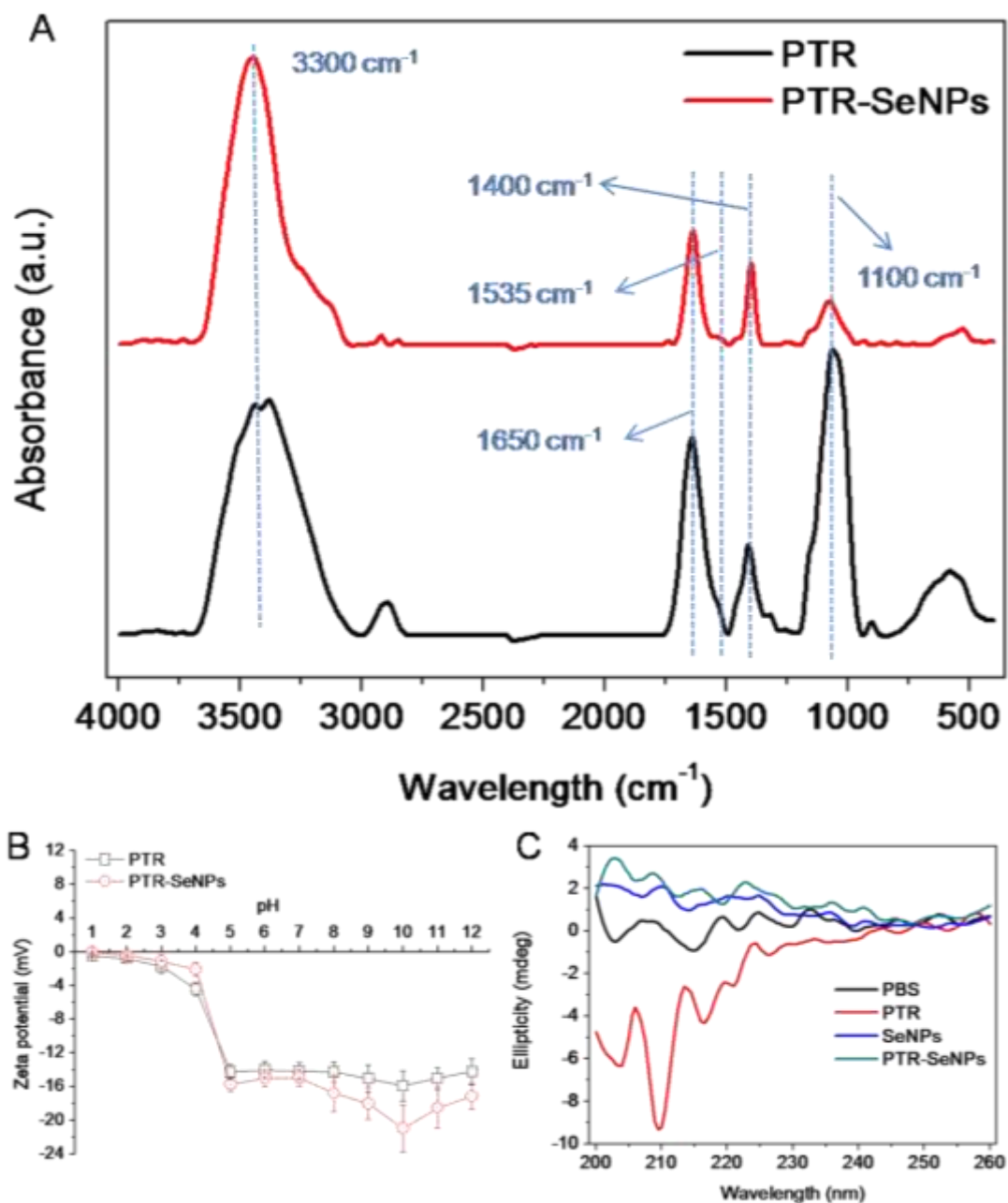
### **Statistical Analysis**

All experiments were conducted in triplicate and results were presented as mean  $\pm$  standard deviation. Except gene expression and mineralization results, which were analyzed by two-tailed Student's *t*-test, all mean values were analyzed by parametric one-way ANOVA followed by multiple comparisons using Turkey-HSD to detect significant differences at  $p < 0.05$ ,  $p < 0.01$   $p < 0.001$  (GraphPad Prism, San Diego, CA, USA).

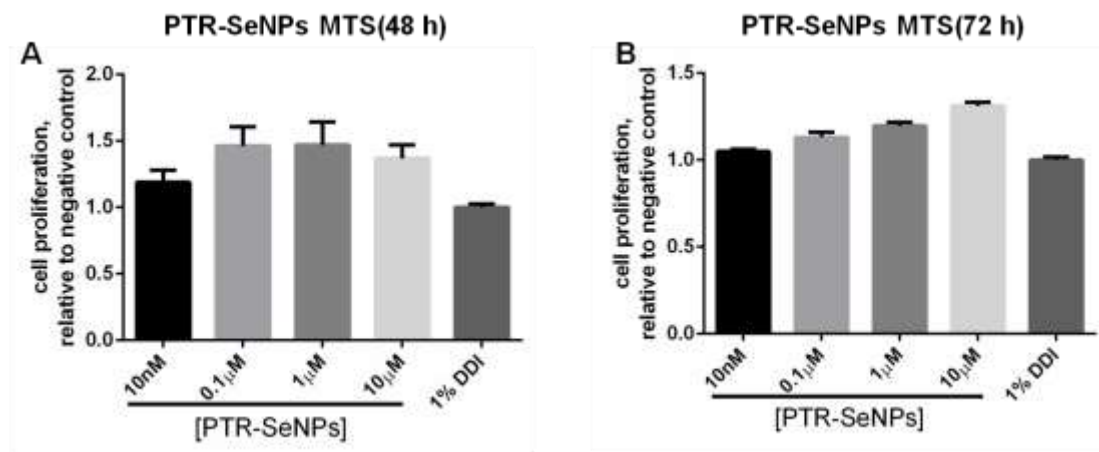
## Results



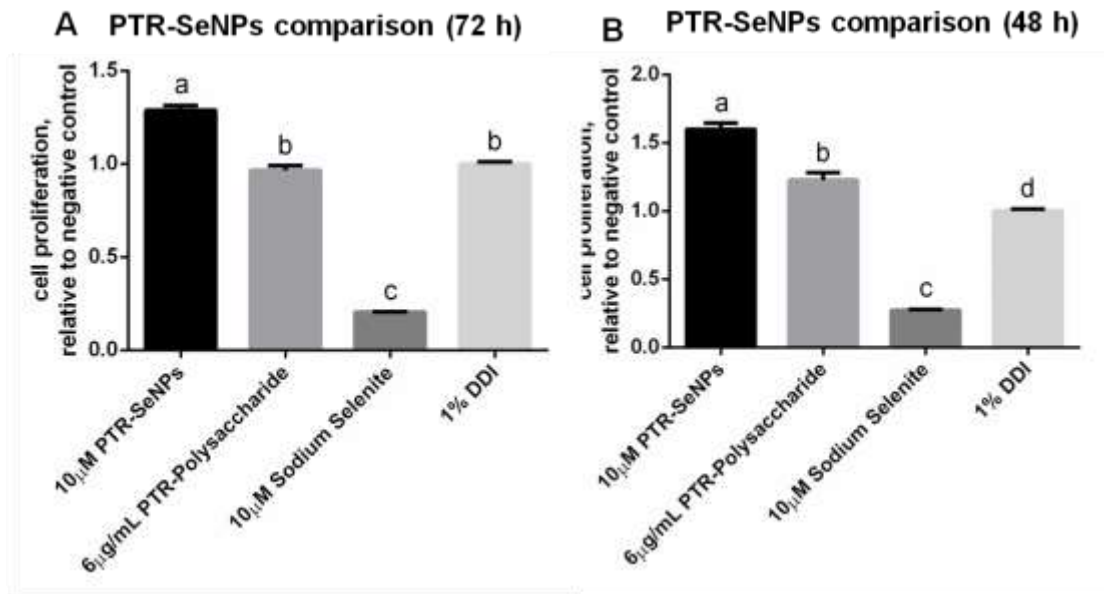
**Fig. S1** Particle size distribution of PTR-SeNPs in aqueous solution measured by Nanosight NS300, showing an average particle size of  $91.3 \pm 1.5$  nm (The polydispersity is 1.6%).



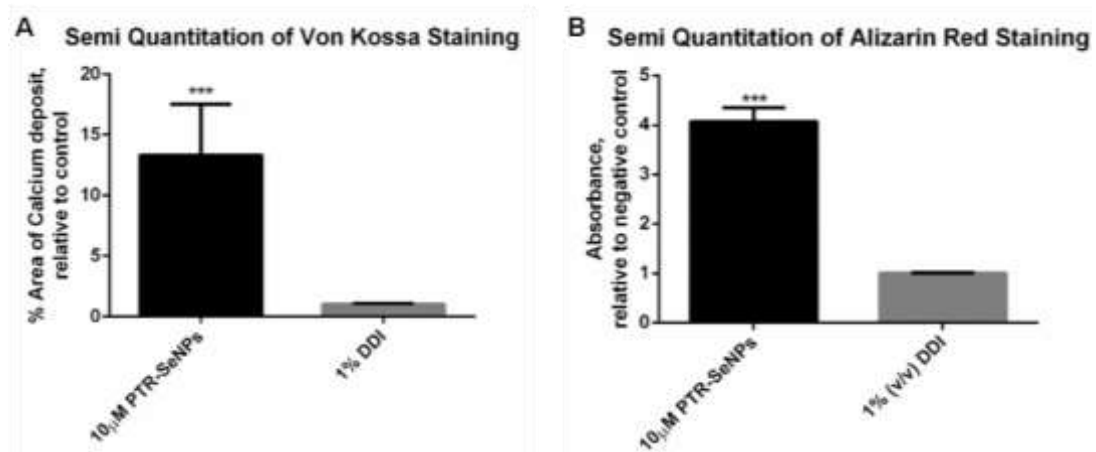
**Fig. S2** A, FT-IR spectra of PTR-PSP and PTR-SeNPs in absorbance mode. B, Evolution of Zeta potential values of PTR and PTR-SeNPs under different pH conditions. C, CD spectra of PBS, PTR, SeNPs, PTR-SeNPs in the wavelength range from 200 nm to 260 nm.



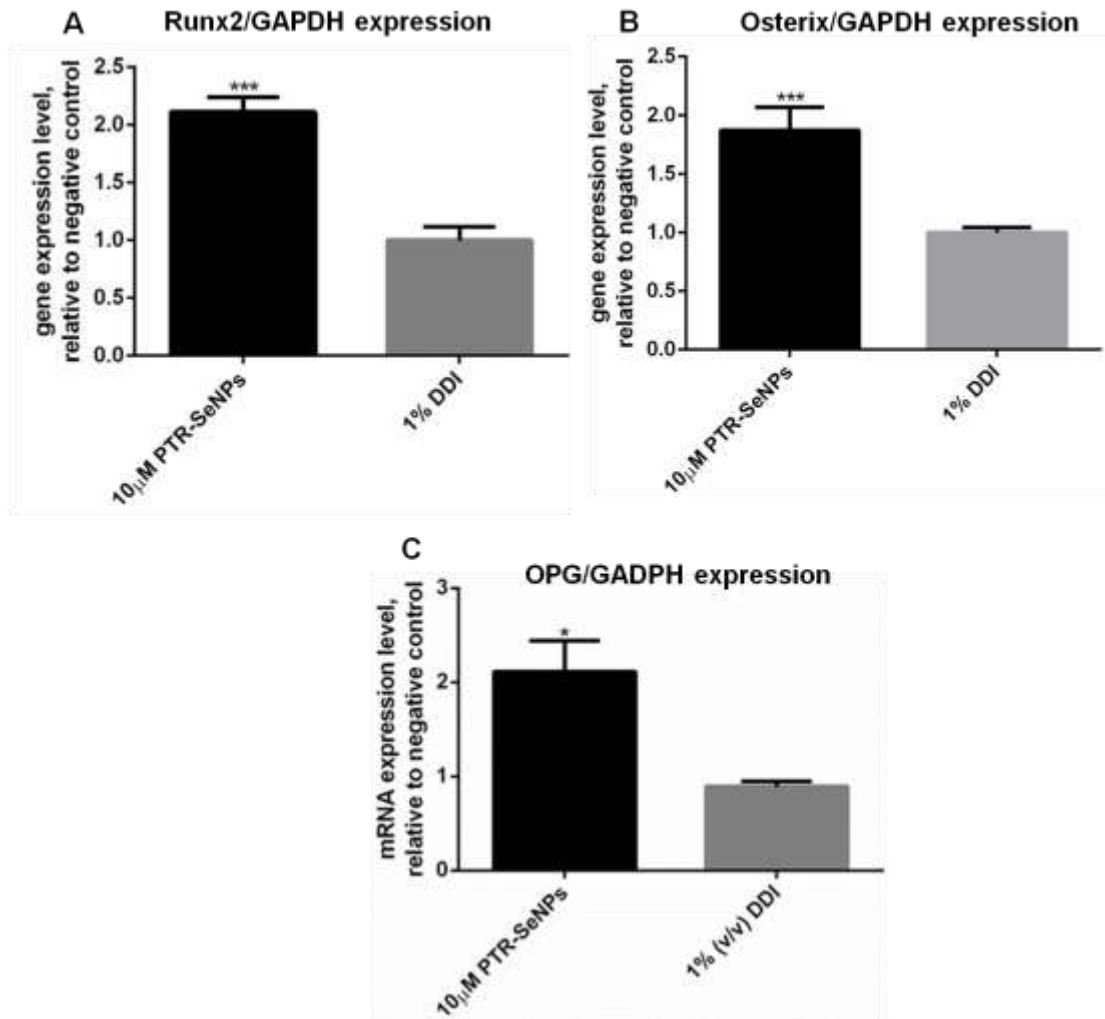
**Fig. S3** Proliferation of MC3T3-E1 cells treated with different concentrations of PTR-SeNPs (10nM - 10µM) in normal medium for (A) 48 h and (B) 72 h.



**Fig. S4** Proliferation of MC3T3-E1 cells treated with PTR-SeNPs (10 $\mu$ M), PTR-PSP (6 $\mu$ g/mL) and Na<sub>2</sub>SeO<sub>3</sub> (10 $\mu$ M) in normal medium for (A) 48 h and (B) 72 h.

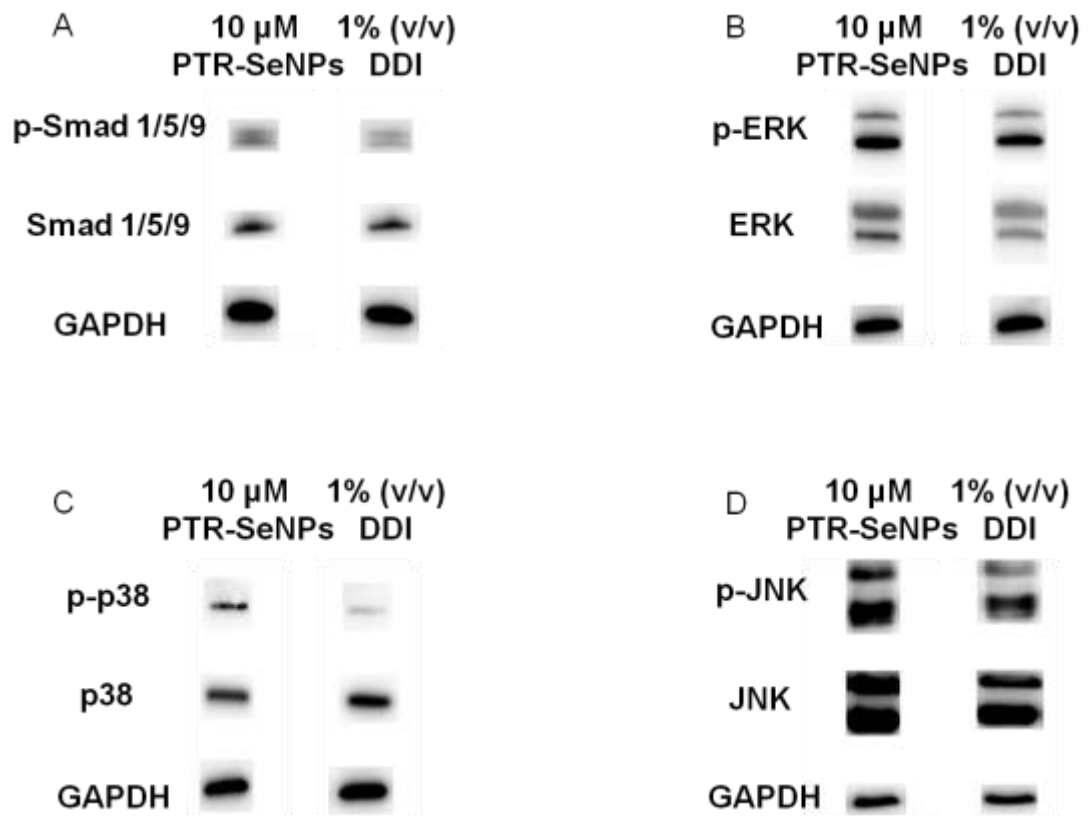


**Fig. S5** A, Semi-quantification of Von Kossa staining by ImageJ. B, Semi-quantification of Alizarin Red S staining by spectrophotometry.

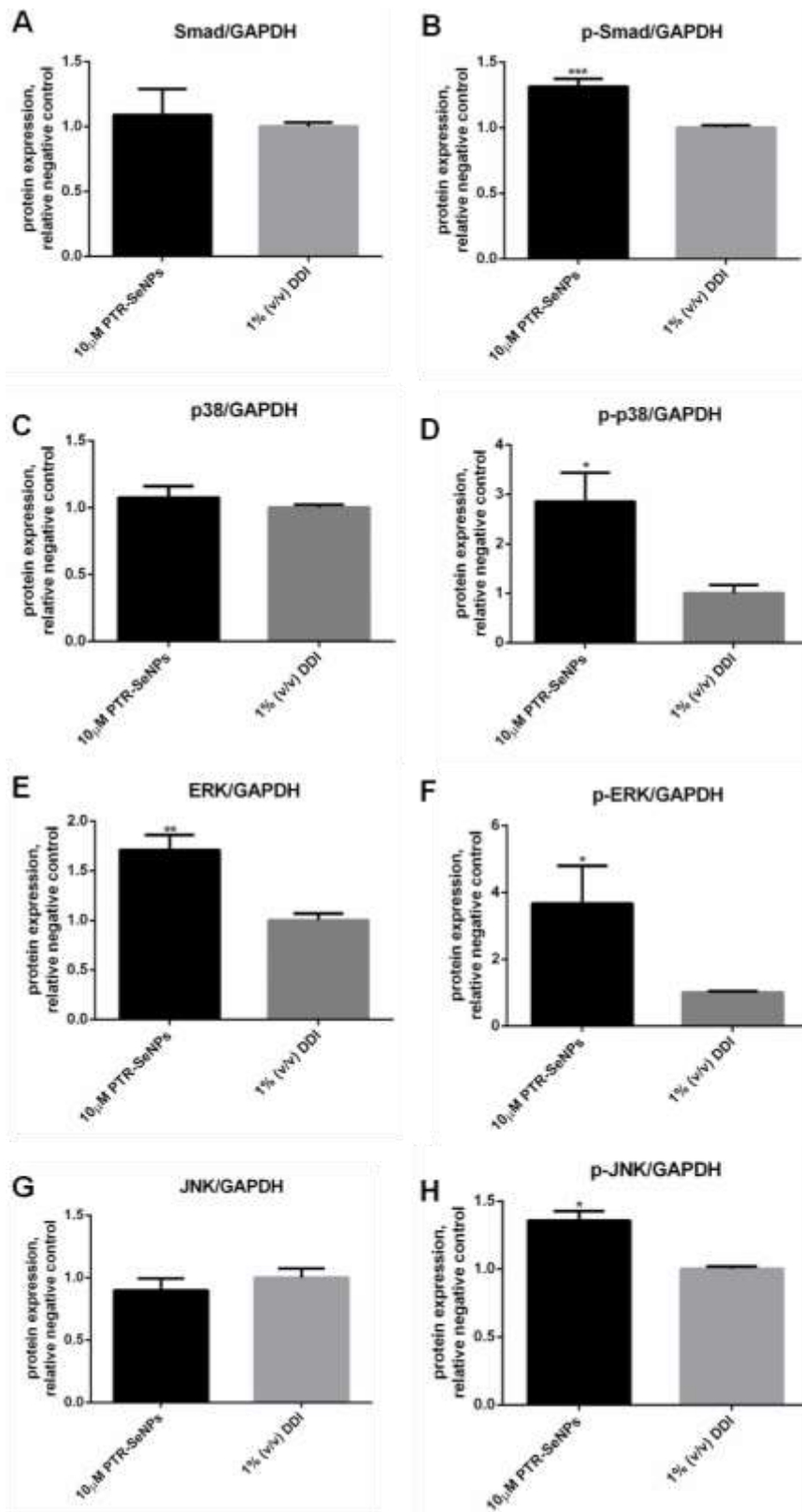


**Fig. S6** Gene expression of (A) Runx2, (B) Osx, (C) OPG/RANKL in MC3T3-E1 cells pre-treated with 10 $\mu$ M of PTR-SeNPs or 1% (v/v) DDI for 3 (Runx2), 11 (OPG/RANKL) and 16 days (OCN), quantified by qPCR analysis and normalized to that of GAPDH.

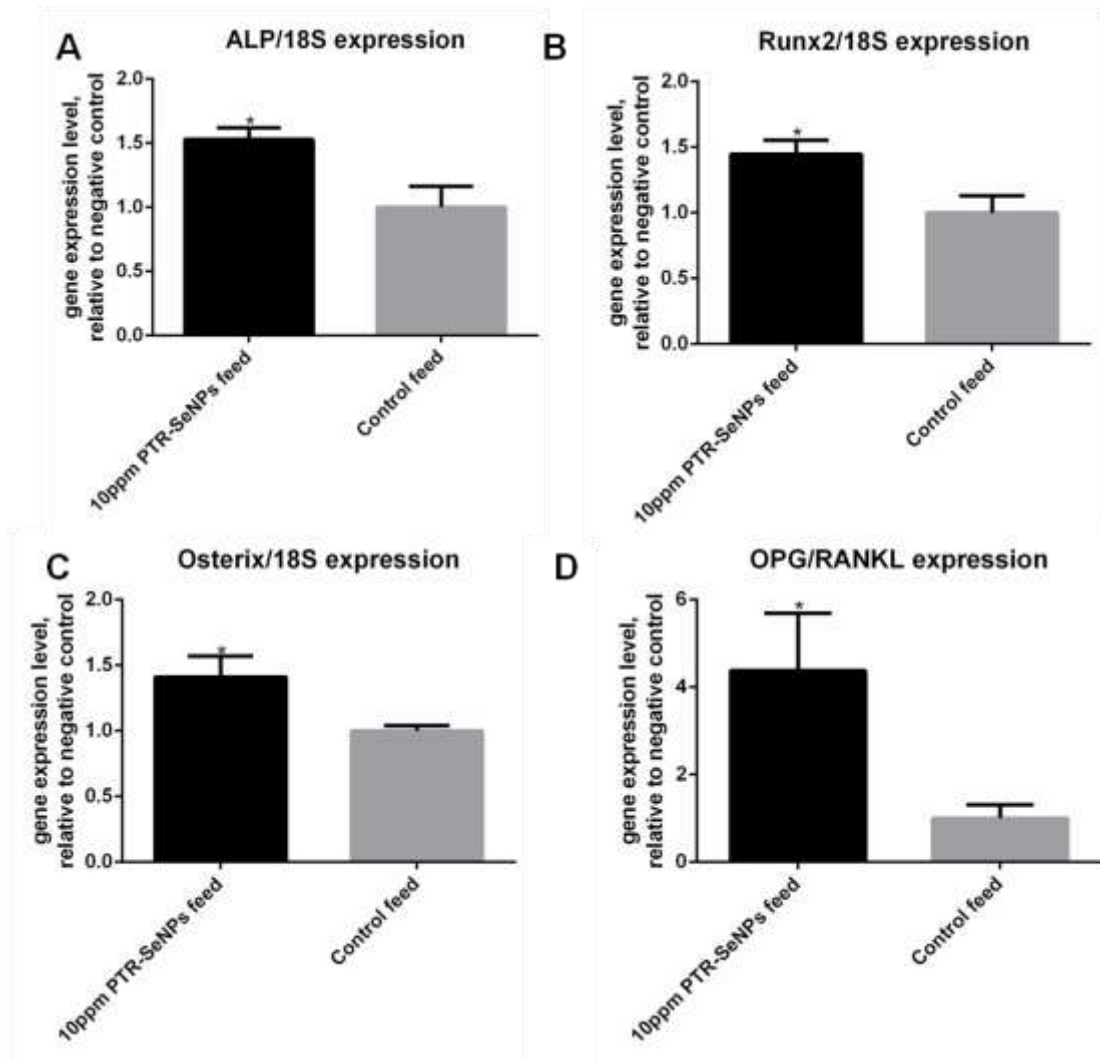




**Fig. S7** Western blot results of protein expression and phosphorylation of (A) Smad1/5/9, (B) ERK, (C) p38 and (D) JNK in MC3T3-E1 cells pre-treated with 10 $\mu$ M of PTR-SeNPs.



**Fig. S8** Semi-quantification of western blot results of Smad1/5/9 expression (A) and phosphorylation Smad1/5/9 (B), p38 expression (C) and phosphorylation p38 (D), ERK expression (E) and phosphorylation ERK (F), JNK expression (G) and phosphorylation JNK (H), all normalized to that of GAPDH.



**Fig. S9** PTR-SeNPs up-regulated the major osteogenic markers in medaka larvae during vertebrae development. Gene expression of (A) ALP, (B) Runx2, (C) Osx and (D) OPG/RANKL in medaka larvae fed with 10ppm of PTR-SeNPs based diet or control diet for 2 days, quantified by qPCR analysis and normalized to that of 18S.