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Low-molecular-weight estrogenic phytoprotein suppresses osteoporosis development through positive modulation of skeletal estrogen receptors

John Akrofi Kubi [a,b], Augustine Suurinobah Brah [a,b], Kenneth Man Chee Cheung [a,b], Andy Chun Hang Chen [c,d], Yin Lau Lee [c,d], Kai-Fai Lee [c,d], Wei Qiao [e], Yibin Feng [f], Kelvin Wai Kwok Yeung [a,b,*]

[a] Department of Orthopaedics and Traumatology, Li Ka Shing Faculty of Medicine, The University of Hong Kong (HKU), PR China
[b] Shenzhen Key Laboratory for Innovative Technology in Orthopaedic Trauma, HKU-Shenzhen Hospital, Shenzhen, 518053, PR China
[c] Department of Obstetrics and Gynaecology, Li Ka Shing Faculty of Medicine, HKU, 21 Sassoon Road, PR China
[d] Shenzhen Key Laboratory of Fertility Regulation, Reproductive Medicine Center, HKU- Shenzhen Hospital, Shenzhen, PR China
[e] Applied Oral Sciences and Community Dental Care, Faculty of Dentistry, PR China
[f] School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong (HKU), PR China

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ABSTRACT

Age-related osteoporosis is a metabolic skeletal disorder caused by estrogen deficiency in postmenopausal women. Prolonged use of anti-osteoporotic drugs such as bisphosphonates and FDA-approved anti-resorptive selective estrogen receptor modulators (SERMs) has been associated with various clinical drawbacks. We recently discovered a low-molecular-weight biocompatible and osteoanabolic phytoprotein, called HKUOT-S2 protein (32 kDa), from *Dioscorea opposita* Thunb that can accelerate bone defect healing. Here, we demonstrated that the HKUOT-S2 protein treatment can enhance osteoblasts-induced ossification and suppress osteoporosis development by upregulating skeletal estrogen receptors (ERs) ERα, ERβ, and GPR30 expressions *in vivo*. Also, HKUOT-S2 protein estrogenic activities promoted hMSCs-osteoblasts differentiation and functions by increasing osteogenic markers, ALP, and RUNX2 expressions, ALP activity, and osteoblast biomineralization *in vitro*. Fulvestrant treatment impaired the HKUOT-S2 protein-induced ERs expressions, osteoblasts differentiation, and functions. Finally, we demonstrated that the HKUOT-S2 protein could bind to ERs to exert osteogenic and osteoanabolic properties. Our results showed that the biocompatible HKUOT-S2 protein can exert estrogenic and osteoanabolic properties by positively modulating skeletal estrogen receptor signaling to promote ossification and suppress osteoporosis. Currently, there is no or limited data if any, on osteoanabolic SERMs. The HKUOT-S2 protein can be applied as a new osteoanabolic SERM for osteoporosis treatment.

1. Introduction

Age-related osteoporosis is the most common metabolic skeletal disorder characterized by bone microarchitectural deterioration, reduced bone mechanical properties, and increased bone fragility leading to recurrent fractures [1–7]. It has been estimated that more than $95 billion will be spent on 3.2 million osteoporosis-related bone fracture treatments in the US by 2040 whereas about $581.97 billion will be spent on 5.91 million hip fracture treatments in China by 2050 annually [8–11]. The alarming rate of global osteoporotic bone fractures coupled with hefty treatment costs has severe health and socioeconomic impacts on the patients and health authorities. There is therefore a very high global demand for cost-effective anti-osteoporotic interventions that can suppress osteoporosis development to improve bone health and alleviate treatment costs. Identification of the principal root causes of osteoporosis will be useful for developing safer anti-osteoporosis therapies. In age-related osteoporosis, estrogen insufficiency and functional impairments of estrogen receptors (ERs) are the main etiological factors that disrupt the homeostatic balance between bone formation and resorption leading to osteoporosis progression [12–17]. Ovaries are the main estrogen-producing organs in females. Hence, estrogen deficiency-induced osteoporosis is more predominant in aged females

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* Corresponding author. Department of Orthopaedics and Traumatology, Li Ka Shing Faculty of Medicine, The University of Hong Kong (HKU), PR China.
E-mail address: wkkyeung@hku.hk (K.W.K. Yeung).

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than males [15,16,18–20] .

Estrogen is a sex hormone that plays a critical role in modulating reproductive functions, secondary sexual characteristics, and bone metabolism in humans [21,22]. Estrogen regulates the functions of osteoblasts and osteoclasts to maintain bone remodeling and homeostasis [22–24]. Osteoblasts are the functional synthesizers of new bone tissues [25]. Consequently, osteoblasts are one of the main cellular targets by which estrogenic stimuli modulate bone metabolism. An *in vitro* study has shown that estrogen treatment enhanced mesenchymal stem cells (MSCs)-derived osteoblasts differentiation and survival [24]. Co-treatment of estrogen with BMP-2 protein promoted osteoblast differentiation by increasing osteoblastic gene expressions, enhancing ALP activity, and biomineralization *in vitro* [26,27]. Estrogen can also alter osteoclast functions by reducing osteoclast number, differentiation, and bone resorption capabilities [19,22,23,28,29]. Many animal studies have proven that ovariectomized (OVX) animals develop osteoporosis due to estrogen deficiency-induced bone loss [6,7,17,30–34]. Understanding the interaction between estrogenic stimuli and osteoblasts can provide opportunities for developing estrogenic therapies for treating estrogen-deficient bone disorders such as osteoporosis.

Estrogen binds to the estrogen receptors (ERs) (ERα, ERβ, and GPR30) that are differentially expressed in osteoblasts and osteoclasts to modulate bone metabolism [16,31,32,35–43]. Suppression of ERs expressions corresponded to bone loss in both menopausal women and OVX mice [16,44]. Furthermore, it has been demonstrated that osteoblasts lineage-specific ERα depletion reduced cortical bone mineral density [45]. Also, osteoclasts lineage-specific ERα deletion stimulated osteoclastogenesis and bone resorption leading to deteriorated trabecular bone microarchitecture [45,46]. Depletion of ERs in mice (ERα−/− or ERαβ double-knockout ERαβ−/−) impaired the remodeling of cortical or trabecular bone microarchitecture leading to reduced bone mass in both male and female mice [16,44]. All these studies clearly indicated that both estrogenic stimuli and ERs suppression contribute to the pathogenesis of osteoporosis. Estrogenic stimuli and ERs modulations are therefore therapeutic targets for suppressing osteoporosis development to minimize osteoporosis-associated bone fractures.

The first-choice anti-osteoporotic drugs such as bisphosphonates (alendronate, risedronate) have been linked with severe musculoskeletal pains, jawbone osteonecrosis, femoral cracks, and the risk of esophageal cancer [13,47,48]. Clinical studies have shown that estrogen replacement therapy (ERT) can help improve bone microarchitecture to minimize the incidence of osteoporotic bone fractures in postmenopausal women [13,49–52]. However, ERT-associated clinical side effects such as risks of developing cardiovascular diseases, breast cancers, and stroke have hindered its clinical application [13,53,54]. Consequently, clinical studies have shifted towards the use of selective estrogen receptor modulators (SERMs) which selectively bind to ERs to suppress osteoporosis [13,15,51,55,56]. The application of SERMs such as raloxifene and bazedoxifene helped minimize the clinical setbacks of ERT in osteoporosis treatment. However, SERMs have also been reported to induce muscle clamps, stroke, and thromboembolic disorders in osteoporotic patients [13]. This has necessitated the development of new biocompatible SERMs for osteoporosis treatment. Development of biocompatible osteoanabolic biomaterials that modulate skeletal ERα, ERβ, and GPR30 to maintain bone mass and microarchitecture can, therefore, be clinically applicable in suppressing bone mass deterioration under osteoporotic conditions. Clinical application of skeletal ERs modulators can also help reduce the risk of osteoporotic bone fractures.

Various forms of bioactive materials have been used to prevent osteoporotic bone loss *in vivo*. For instance, it was reported that fabricated caffeic acid nanospheres and deferoxamine-coated titanium implants promoted ossification and prevented bone loss under osteoporotic conditions [57]. Another study revealed that tail intravenous injection of 50 μg/mL bone targeting fluffy surface hybrid nano-adjuvant loaded with alendronate modulated bone remodeling processes to prevent osteoporotic bone loss [58]. In addition, aqueous administration of 200 mg/kg/day LiCl was reported to have suppressed bone resorption but enhanced ossification in osteoporotic alveolar bone [59]. It was also reported that aqueous administration of combined 5 mg/kg dasatinib and 50 mg/kg quercetin suppressed postmenopausal osteoporotic bone loss in rats [6]. Recently, our group discovered a low-molecular-weight HKUOT-S2 protein (32 kDa) from *Dioscorea opposita* Thunb and demonstrated the HKUOT-S2 protein can modulate macrophage-MSCs crosstalk and osteoblast functions to promote osteogenesis and bone defect repairs [25]. The HKUOT-S2 protein is biocompatible and exhibits no toxic effects on blood, liver, kidney, spleen, heart, and brain tissues [25]. In the current study, we demonstrated that the HKUOT-S2 protein can suppress osteoporosis progression through positive modulation of skeletal ERα, ERβ, and GPR30 expressions to promote osteogenesis and facilitate osteoblast-induced bone formation (see Scheme 1). The HKUOT-S2 protein can be potentially applied as a new biocompatible osteoanabolic SERM for treating osteoporosis.

2. Materials and methods

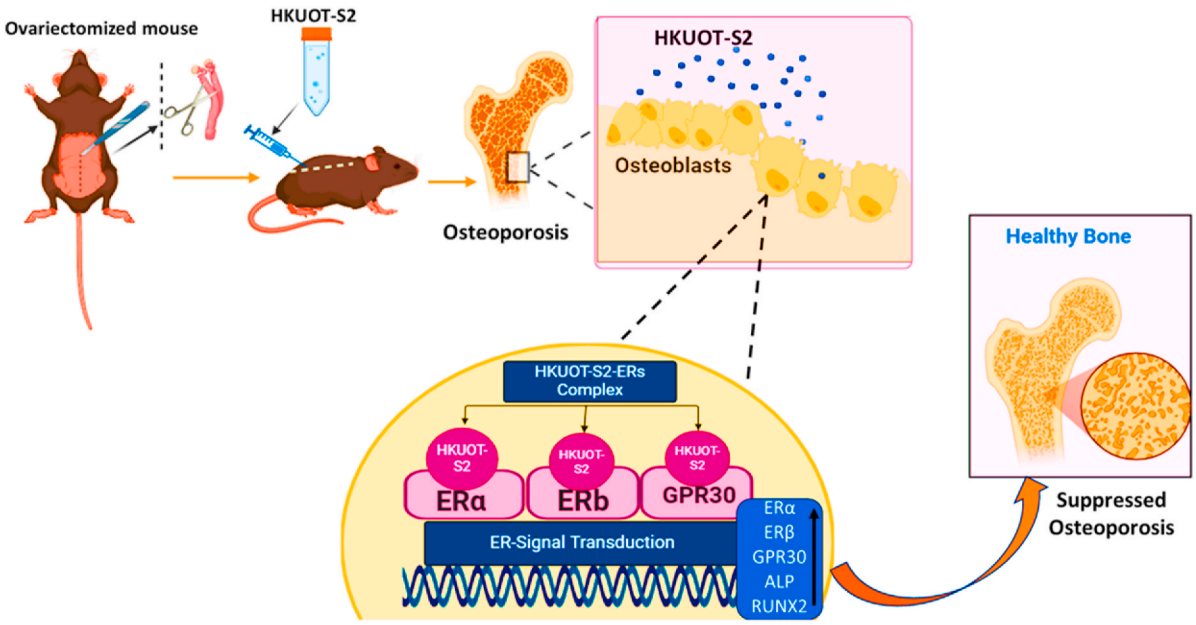
2.1. Animal studies

Women have a higher risk of developing age-related osteoporosis than men [60,61]. Estrogen deficiency is one of the major causes of postmenopausal osteoporosis development [61]. As the ovaries are the main estrogen-synthesizing organs in females, ovariectomized female mice were used in this study to induce and mimic postmenopausal osteoporotic conditions.

All the animal housing and experimental procedures were strictly performed in accordance with the approved protocol by the HKU Ethics Committee, Committee on the Use of Live Animals in Teaching and Research (CULATR), (CULATR 5843–21). A total of 96C57BL/6J female mice were used in this study. The female mice aged between 8 and 10 weeks and weighing between 20 and 22g were obtained from The Centre for Comparative Medicine Research (CCMR), HKU. The mice were randomly assigned into four groups namely the sham control (n = 16), OVX control (n = 16), OVX+ 30 μg/kg β-estradiol (E2) (n = 16), and OVX+2.18 mg/kg HKUOT-S2 (n = 16) treatment groups. In summary, all the mice were anesthetized, placed firmly in a prone position, and subjected to a dorsoventral surgical operation. The bilateral furs over the lumbar spine (flank area, between the last rib and above the pelvis) were shaved and the exposed skin was cleansed with betadine and 70 % ethanol four times sequentially. A 0.5 cm single midline dorsolateral incision (on the right side) was made on the lumbar spine at the lower back just below the bottom of the rib cage. Blunt forceps were used to gently separate the subcutaneous connective tissue from the underlying muscle on either side. The ovaries were located and completely removed from the anterior uterine horns in the OVX treatment groups (OVX control, OVX + 30 μg/kg E2, and OVX + 2.18 mg/kg HKUOT-S2). The anterior uterine horns were tied with a suture clip (4/0 Ethilon) in the OVX groups. The sham control mice also underwent surgery without ovary removal or tying of the anterior uterine horns. The wounds were closed by systematic suturing of the muscles, subcutaneous, and superficial skin layers using 5/0 vicryl®-coated filament and 4/0 Ethilon. The mice were given intraperitoneal injections (i.p) of 200 μl 1X PBS for the sham and OVX controls, 30 μg/kg E2 for the E2 group, and 2.18 mg/kg HKUOT-S2 for HKUOT-S2 group immediately after surgery and thrice per week for one month. The femurs and lumbar vertebrae were harvested and processed for immunostaining, Western blot, histological, ALP activity, and qPCR analyses.

2.2. Micro-computed tomography (μCT) scan analyses of the femur and lumbar vertebrae

The μCT scans of the femur and Lumbar vertebrae of the anesthetized mice were performed immediately after the surgery (day zero) and at the experimental endpoint using the Skyscan (Bruker). The NRecon



Scheme 1. Proposed biological mechanisms of HKUOT-S2 protein. Schematic diagram illustrating that HKUOT-S2 protein suppressed osteoporosis development through estrogen receptor signaling.

software (Skyscan, Bruker) was used to reconstruct 2D images of the μCT scans. The femur and Lumbar vertebrae morphometric parameters such as percentage bone volume (BV/TV), bone surface area to tissue volume (BS/TV) ratio, trabecular thickness (Tb.th), and trabecular number (Tb. N), were assessed in the sham control and OVX treatment groups using the CTvox and CTAn software.

2.3. Immunofluorescence staining analysis

Harvested mouse bone samples and hMSCs-derived osteoblasts were histologically and cytologically processed according to the protocol used in our previous work [25]. The fixed cells or 4–6 μm paraffin-embedded bone sections were then processed for immunofluorescence staining using ERα, ERβ, GPR30, ALP, and RUNX2 antibodies (Table S1) according to immunofluorescence staining standard protocol [25].

2.4. Western blot analysis

The total proteins isolated from the lysed cells and bone samples were measured with the Pierce Bradford Plus Protein Assay kit (Thermo Scientific, A55866) (Table S4) and subjected to SDS-PAGE analyses [25]. The separated proteins in the gel were transferred onto PVDF membranes. The membranes containing the proteins were blocked with 5 % BSA (GoldBio, A-420-250) in 1X PBST solution and incubated with the appropriate primary antibodies (Table S1) in a blocking buffer for 24 h at 4 °C. The membranes were washed with 1X PBST followed by 2 h of incubation with appropriate secondary antibodies (Table S1) at room temperature. The washed membranes were probed with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, 32209) (Table S4) followed by protein band analysis with GE Amersham Imager AI680 [25].

2.5. ALP activity assay

The bone tissue and cell sample ALP activity tests were performed using the alkaline phosphatase kit (Beyotime, P0321M) and 1-step NBT/BCIP solution (Thermo Scientific, 34042) according to the manufacturers' instructions.

2.6. Quantitative polymerase chain reaction (qPCR) analysis

The total pure RNAs extracted from the bone and cultured cell samples were processed for qPCR gene analyses using appropriate kits and gene primer pairs (Table S2 and Table S3) according to our previously published protocol [25]. Briefly, the extracted RNAs with RNAiso plus reagent (Takara, 9109) were converted into complementary DNA (cDNA) using the PrimeScript™ RT reagent Kit (Takara RR036A). The reaction mixture containing the cDNA and appropriate primer pairs for SYBR green was used to determine the mRNA levels using the QuantStudio 5 real-time PCR system (Thermo Scientific, A34321). Gene expressions were normalized with the housekeeping gene, *GAPDH*. The results were calculated as fold changes relative to the control using the $2^{-\Delta\Delta Ct}$ method.

2.7. Tartrate-resistant acid phosphatase (TRACP) and alkaline phosphatase (ALP) immunohistochemistry (IHC) staining

The 4–6 μm of histologically processed bone tissues from the sham control and OVX mice were subjected to TRACP/ALP IHC staining using a TRACP & TRAP double stain kit (Takara, MK300) according to the manufacturer's instructions followed by fluorescence microscopy analyses [25].

2.8. Transmission electron microscopy (TEM) analysis

A 2 mm thickness of the bone tissues from the sham control and OVX mice was processed for TEM analysis at The University of Hong Kong Electron Microscopy Unit followed by bone tissue microarchitectural analyses with Philips CM100 TEM model.

2.9. In vivo skeletal fluorochrome labeling

The experimental mice were given 10 mg/kg Calcein green (Sigma-Aldrich, C0875-5G) and 90 mg/kg xylenol orange (Sigma-Aldrich, X-0127) ten and three days respectively before euthanasia. The harvested bone tissues were histologically processed for confocal fluorescence microscopy analyses using Carl Zeiss (LSM800) equipped with the ZEISS software according to the previously published protocol [25].

2.10. H&E staining

The bone tissues from the sham control and OVX mice were histologically processed, and paraffin embedded to conserve the architecture of the bone tissues for downstream histological analyses. 4–6 μm of the bone tissue sections were further processed for H &E staining for light microscopy analysis according to a published protocol [25].

2.11. Human turbinate mesenchymal stromal cell line (hMSCs)-osteoblasts differentiation

The hMSCs produced by Kwon et al. [62] were maintained in DMEM low glucose medium (Gibco, 11885-076) containing 10 % FBS and 100 U/ml Penicillin-Streptomycin (P/S) within humidified incubators at 37 °C and 5 % CO_2. The hMSCs were differentiated into osteoblasts according to the previously published protocol [25] with or without 10 μM fulvestrant, 10 nM E2, or 3.125 nM HKUOT-S2 protein treatments.

2.12. Alizarin Red S (ARS) staining

4 % paraformaldehyde-fixed cells were processed for ARS staining (Sigma-Aldrich, A5533-25G) to evaluate osteoblast biomineralization according to the previously published protocol [25].

2.13. Silver staining

The HKUOT-S2 protein in the SDS-PAGE gels was subjected to silver staining using the Pierce Silver Staining for Mass Spectrometry kit (Thermo Scientific, 24600) according to the manufacturer's protocol.

2.14. Immunoprecipitation (IP) assay

The HKUOT-S2 protein dissolved in ultra-pure water was precipitated from the solution by ERα, ERβ, and GPR30 antibodies using the Pierce Co-Immunoprecipitation kit (Thermo Scientific, 26149) according to the manufacturer's protocol. The HKUOT-S2 protein-ERs complexes in the IP elutes were separated in SDS-PAGE gel followed by silver staining analysis.

2.15. Statistical analysis

Turkey's test post-hoc comparisons for normal distribution and Kruskal-Wallis post-hoc comparisons for non-parametric tests of One-way ANOVA were employed to analyze the data in GraphPad Prism 8.0 (GraphPad Software, CA, USA). The values were expressed as mean ± SEM with significant *$p < 0.05$.

3. Results

3.1. HKUOT-S2 protein suppressed OVX-induced osteoporosis development

Animal studies have shown that OVX can induce osteoporosis in the femur and lumbar vertebrae due to estrogen deficiency [63–67]. In our recently published work, we demonstrated that HKUOT-S2 protein could modulate osteoblast functions to promote osteogenesis and bone defect repairs [25]. We, therefore, anticipated the HKUOT-S2 protein to exhibit anti-osteoporotic properties to maintain physiological bone health under osteoporotic conditions. To test the anti-osteoporotic potential of the HKUOT-S2 protein, C57BL/6J female mice were subjected to ovariectomy (OVX) to induce age-related and estrogen-deficient osteoporosis that mimicked postmenopausal osteoporotic condition (Fig. 1A). The effects of HKUOT-S2 treatment on femoral and vertebral bones were assessed because it was estimated that the rate of osteoporotic femoral or vertebral bone fractures globally occur every 3.33 min [60]. The μCT scan analyses showed that HKUOT-S2 protein treatment suppressed femoral and vertebral osteoporosis development when compared with the OVX control (Fig. 1B, Fig. S1A, C, E). Morphometric analyses of the femoral and vertebral bone microarchitectures showed that HKUOT-S2 protein significantly increased percentage bone volume (BV/TV), bone surface area to tissue volume (BS/TV) ratio, trabecular thickness (Tb.th) and trabecular number (Tb. N) when compared with the OVX control group (Fig. 1C, Fig. S1B, D, F). The HKUOT-S2 protein treatment also maintained femoral and vertebral bone architectures as that of the sham and E2 (positive) controls. The results were supported by another study which showed that estrogen treatment 5 times per week suppressed bone loss in OVX C57BL/6J mice [63]. The data suggested that HKUOT-S2 protein might possess estrogenic properties, similar to that of the E2 treatment, to suppress osteoporosis progression in the OVX mice. Bone histological analysis by H&E staining showed that femoral cortical bone thicknesses were well conserved in the sham control, E2, and HKUOT-S2 protein treatment groups compared to the OVX control with thinner cortical bone thickness (Fig. 1D). It has been well-documented that functional osteoblasts synthesize extracellular bone matrix to facilitate new bone formation [25,68]. Masson-Goldner trichrome staining was therefore performed to test whether the HKUOT-S2 protein treatment enhanced osteoblast functions to maintain bone health under OVX-induced osteoporotic conditions. The results demonstrated that HKUOT-S2 protein treatment, indeed, induced osteoblasts to synthesize and deposit red-staining mature bone matrix when compared with the OVX control (Fig. 1E). It was also observed that the bone mineralization patterns were evenly distributed across the bone tissues in both sham control and HKUOT-S2 protein treatment groups whereas that of the E2 group was restricted to the outer layer of the cortical bones (Fig. 1E). The results indicated that HKUOT-S2 protein treatment enhanced osteoblast activities *in vivo* to suppress osteoporosis development. Next, *in vivo* skeletal fluorochrome labeling with calcein green was performed to evaluate whether the HKUOT-S2 protein enhanced osteoblasts activities resulted in new bone formation under osteoporotic conditions. As expected, the results confirmed that HKUOT-S2 protein treatment significantly increased new bone formation (increased calcein green fluorescence intensity) relative to the OVX control. There were no significant differences in terms of new bone formation among the sham control, E2, and HKUOT-S2 protein treatment groups (Fig. 1F and G).

3.2. HKUOT-S2 protein modulated osteogenic activities to prevent OVX-induced bone loss

Studies have shown that estrogenic stimuli enhance osteoblast differentiation and function to maintain bone health [16,22,24,35]. Here, we further investigated whether HKUOT-S2 protein treatment enhanced osteoblastic differentiation and activities to prevent osteoporotic bone loss. The bone tissues were subjected to osteogenic gene expression analyses by RT-qPCR. The results showed that HKUOT-S2 protein treatment enhanced osteoblasts differentiation *in vivo* by increasing *Alp, Col1a1,* and *Runx2* expressions when compared to the OVX control (Fig. 2A). Immunofluorescence staining results also revealed that HKUOT-S2 protein treatment significantly increased osteogenic markers ALP and RUNX2 expressions in the bone tissue when compared to the OVX control (Fig. 2B, C, E, F). Osteoblasts functional test analysis revealed that HKUOT-S2 protein treatment significantly increased bone tissue ALP activity when compared to the OVX control (Fig. 2D). Nevertheless, it was not known how the HKUOT-S2 protein treatment might have modulated the osteoclasts and osteoblasts numbers to facilitate the observed new bone formation *in vivo*. TRACP/ALP immunohistochemistry (IHC) and transmission electron microscopy (TEM) analyses of the bone tissues were performed to evaluate the effects of the HKUOT-S2 protein treatment on the osteoclast-like and osteoblast-like cell numbers. The TRACP/ALP, IHC staining, and TEM analyses revealed that HKUOT-S2 treatment did not alter the number of $TRAP^+$ cells (osteoclast-like number) in the bone tissue when compared with

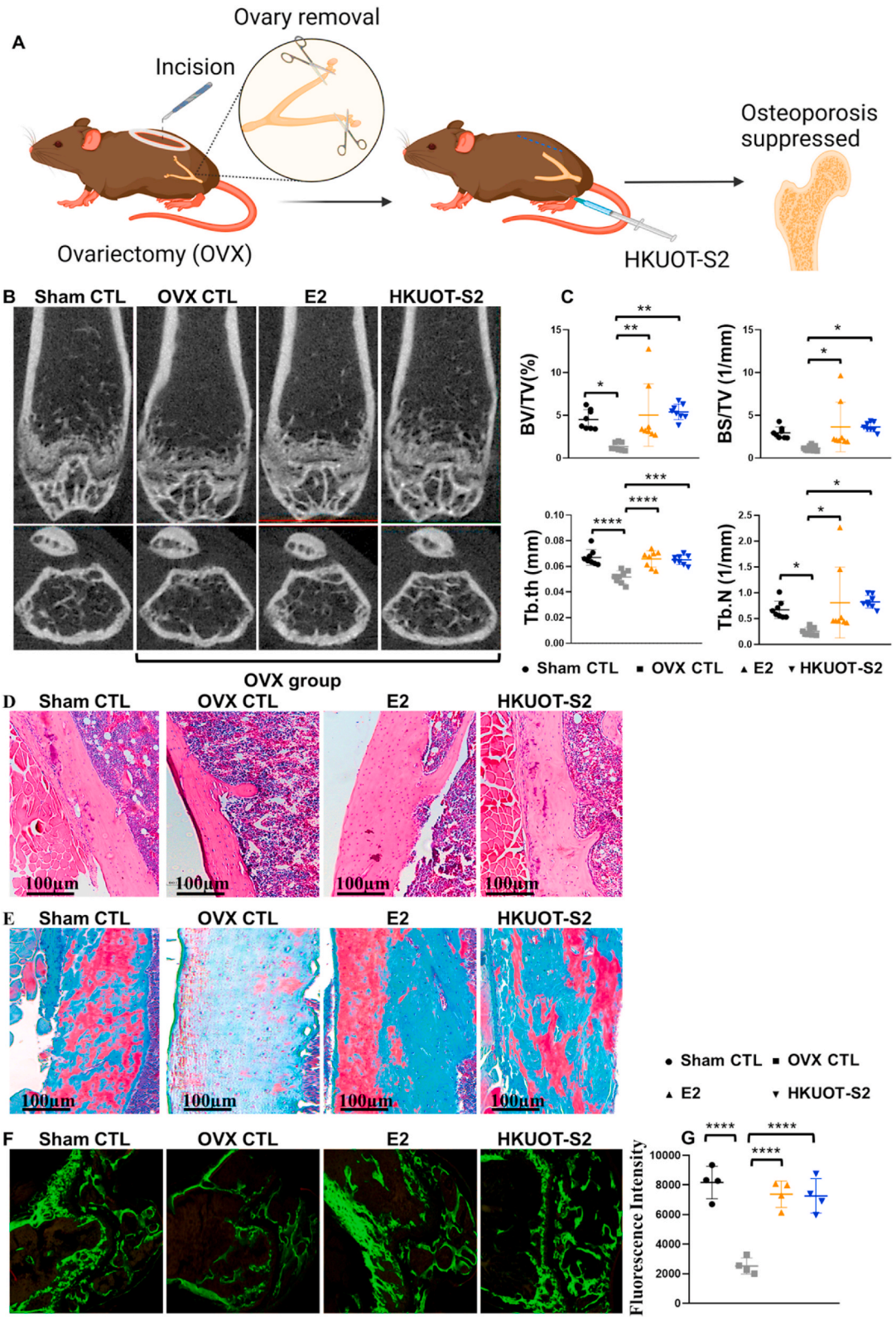


Fig. 1. HKUOT-S2 protein suppressed osteoporosis development of the femur. A) Schematic diagram showing that HKUOT-S2 protein treatment prevented osteoporosis progression in the OVX mouse model. B) Representative μCT scans of the femoral bone. C) μCT analysis of BV/TV, BS/TV, Tb.th, Tb. N of the femoral bone. D) Representative images of H&E staining of bone tissues. E) Representative image of Masson Trichrome staining of the femur. F) Representative image of Calcein green fluorescence intensity of the femur. G) Analysis of Calcein green fluorescence intensity of the femur. The values were shown as mean ± SEM, n = 8 for μCT scan analysis, and n = 4 for calcein green labeling. black scale bars = 100 μm *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

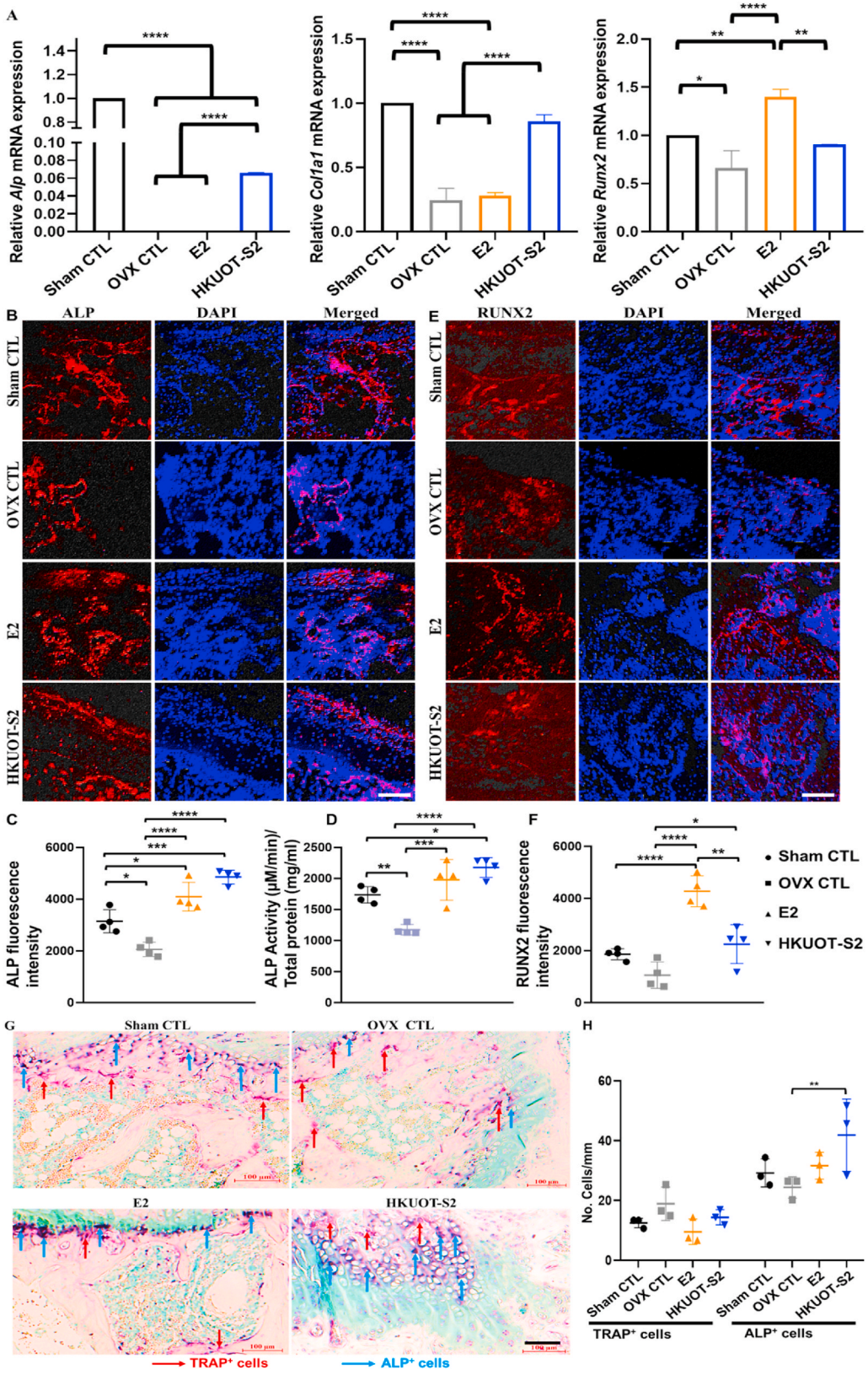


Fig. 2. HKUOT-S2 protein promoted osteoblasts differentiation and activities to prevent bone loss in OVX mice. A) HKUOT-S2 protein treatment increased osteogenic markers *ALP, COL1A1,* and *RUNX2* expressions. B, C) Representative immunofluorescence staining images and quantified fluorescence intensities of ALP protein expression in the experimental mice. D) Quantified bone tissue ALP activity in experimental mice. E, F) Representative immunofluorescence staining images and quantified fluorescence intensities of RUNX2 protein expression in experimental mice G) Representative image of TRACP/ALP staining of the femur. H) Quantification of TRAP + cells and ALP + cells. The values were shown as mean ± SEM. n = 3 for qPCR data and TRACP/ALP staining analyses, n = 4 for immunofluorescence staining, white scale bars = 50 μm, black scale bars = 100 μm, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

the sham control (Fig. 2G, H, Figs. S2A and C). It was observed that TRAP$^+$ cell numbers increased and decreased in the OVX control and E2 treatment group respectively (Fig. 2G and H) which were consistent with other research reports [13,15,19,22,28,69]. HKUOT-S2 protein treatment, however, significantly increased osteoblast-like cell (ALP$^+$ cells) numbers when compared to the OVX control (Fig. 2G, H, Figs. S2A and B). These results were also consistent with another study which reported that OVX decreased osteoblasts number whereas E2 treatment prevented the OVX-induced osteoblasts apoptosis in mice [70]. Taken together, the current results indicated that the HKUOT-S2 protein treatment could enhance osteoblast functions as E2 treatment to maintain physiological bone health and prevent OVX-induced bone loss.

3.3. HKUOT-S2 protein treatment positively modulated skeletal estrogen receptors (ERs) to suppress osteoporosis

Further analysis of the transcriptome data of our previous research work on the bone defect model showed that HKUOT-S2 treatment significantly enriched the estrogen receptor signaling pathway to promote osteogenesis in non-osteoporotic male mice (Fig. S3A) [25]. In the

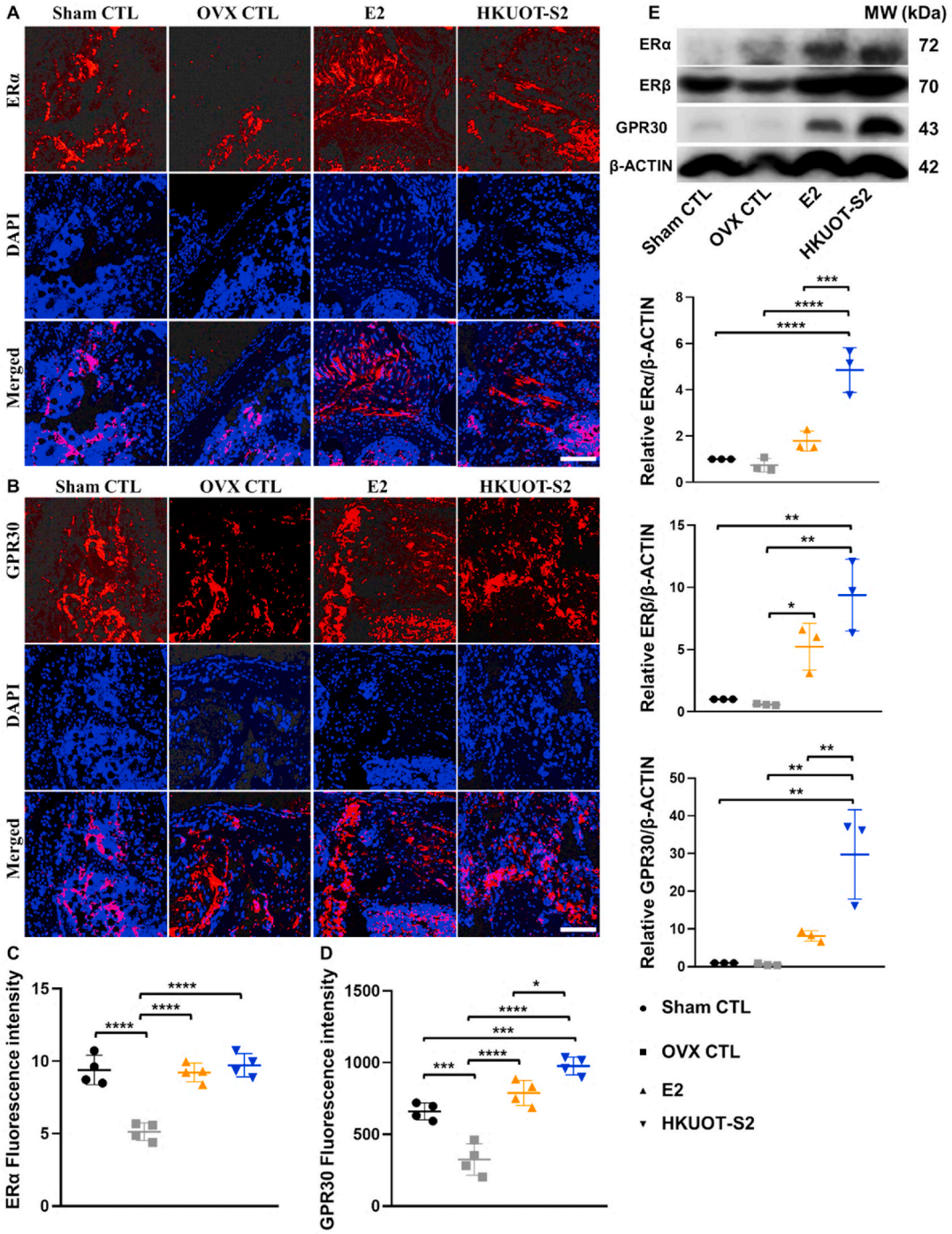


Fig. 3. HKUOT-S2 protein upregulated estrogen receptors (ERs) in the experimental mice to prevent osteoporosis. A-D) Representative immunofluorescence staining images and quantified fluorescence intensities of estrogen receptors ERα and GPR30 in the experimental mice. E) Western blot analyses of ERα, ERβ, and GPR30 in experimental mice. The values were shown as mean ± SEM, n = 4 for immunofluorescence staining, n = 3 for Western blot, white scale bars = 50 μm, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

current study, the HKUOT-S2 protein treatment suppressed osteoporosis development in estrogen-deficient (OVX) mice as that of the E2 (positive) control. We, therefore, anticipated HKUOT-S2 protein treatment to exhibit estrogenic activity. To assess the estrogenic potential of the HKUOT-S2 protein in maintaining bone integrity under OVX-induced osteoporotic conditions, the effects of the HKUOT-S2 protein treatment on skeletal ERα, ERβ, and GPR30 protein expressions were analyzed by immunofluorescence staining and Western blot. The results showed that HKUOT-S2 treatment significantly upregulated the ERα, ERβ, and GPR30 protein expressions in OVX mice to prevent osteoporosis development when compared with the OVX control (Fig. 3A–E, Figs. S3B and C). These results were supported by other studies which demonstrated that activation of ERs by estrogenic stimuli facilitated the maintenance of bone metabolism and served osteoprotective functions [16,19,32,45,71]. The HKUOT-S2 protein estrogenic activities might have promoted osteoblast functions to maintain bone health under OVX-induced osteoporotic conditions. The current findings are consistent with the reports that estrogenic stimuli can modulate osteoblast functions via ERs to maintain bone metabolism [22,70]. Taken together, the results indicated that the HKUOT-S2 protein is as potent as the E2 treatment and can positively modulate skeletal ERs to suppress osteoporosis progression in OVX mice.

3.4. *HKUOT-S2 protein modulated ERs during osteoblasts differentiation in vitro*

It has been reported that E2 treatment increased ERα gene expression during osteoporotic patient-derived MSCs-osteoblasts differentiation [35]. To further assess the effects of HKUOT-S2 protein treatment on osteoblastic ERs gene and protein expressions *in vitro*, hMSCs were

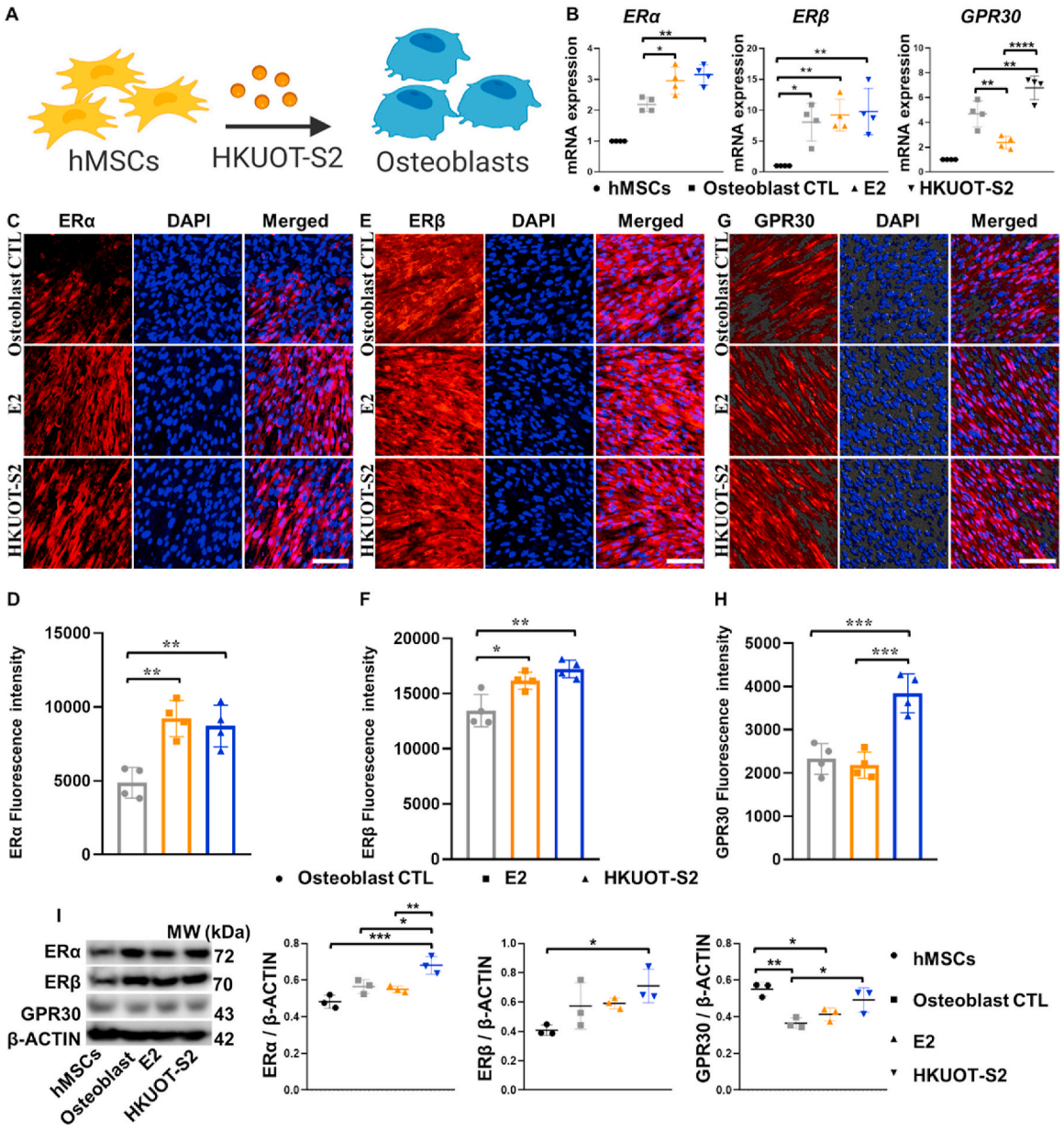


Fig. 4. HKUOT-S2 protein modulated ERs during hMSCs-osteoblasts differentiation. A) hMSCs-osteoblasts differentiation w/o HKUOT-S2 protein. B) Relative mRNA expressions of *ERα, ERβ*, and *GPR30* during hMSCs-osteoblasts differentiation. C-H) Representative immunofluorescence staining images and quantification of ERα, ERβ, and GPR30 protein expressions in osteoblasts. I) Western blot analyses of ERα, ERβ, and GPR30 expressions relative to β-ACTIN during hMSCs-osteoblasts differentiation. n = 4 for qPCR and immunofluorescence staining, n = 3 for Western blot, white scale bars = 50 μm. The values were shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

differentiated into osteoblasts with or without (w/o) E2 or HKUOT-S2 protein (Fig. 4A). The qPCR analyses showed that both E2 and HKUOT-S2 protein treatments significantly increased osteoblastic *ERα* expression but had no effects on *ERβ* expressions when compared with the osteoblasts control (Fig. 4B). The HKUOT-S2 protein treatment significantly increased *GPR30* expression whereas E2 treatment decreased *GPR30* expression when compared with the osteoblasts control (Fig. 4B). Immunofluorescence (IHC) staining results also showed that both HKUOT-S2 protein and E2 treatments significantly increased ERα and ERβ protein expressions when compared to the osteoblasts control (Fig. 4C–F). However, the HKUOT-S2 protein significantly increased GPR30 expression when compared with the osteoblasts control and E2 treatment groups (Fig. 4G and H). Western blot analyses revealed that HKUOT-S2 protein significantly increased the ERα, ERβ, and GPR30 protein expressions when compared to the osteoblasts control (Fig. 4I). The E2 treatment consistently decreased osteoblastic GPR30 protein expression (Fig. 4I). The results demonstrated that both E2 and HKUOT-S2 proteins similarly modulated ERα and ERβ protein expressions but differentially induced GPR30 protein expression during hMSCs-osteoblasts differentiation. The results were consistent with the report that E2 treatment upregulated *ERα* expression during osteoporotic patient-derived MSCs-osteoblasts differentiation [35]. These results were also supported by the report that E2 treatment increased ERα but decreased GPR30 expressions in mouse uterus [72]. This *in vitro* data supplemented the *in vivo* results to confirm that HKUOT-S2 protein could exert estrogenic effects on osteoblasts by upregulating osteoblastic ERs expressions.

3.5. Estrogenic activities of HKUOT-S2 protein enhanced osteoblasts differentiation and functions in vitro

Estrogen treatment has been reported to enhance MSCs-osteoblast differentiation [24]. To evaluate the estrogenic effects of HKUOT-S2 protein on osteoblasts differentiation, hMSCs were differentiated into osteoblasts w/o E2 or HKUOT-S2 protein. The qPCR results confirmed that both HKUOT-S2 protein and E2 treatments enhanced osteoblasts differentiation by significantly increasing *ALP, COL1A1,* and *RUNX2* expressions in the hMSCs-derived osteoblasts when compared to the hMSCs and osteoblast controls (Fig. 5A). Immunofluorescence staining and Western blot analyses revealed that HKUOT-S2 protein enhanced osteoblasts differentiation by significantly increasing osteogenic markers ALP and RUNX2 expressions when compared to that of the osteoblast control and E2 treatment groups (Fig. 5B–F). Osteoblastic functional tests were conducted to verify whether the HKUOT-S2 protein-induced osteogenic gene transcriptions and protein translations could enhance osteoblast functions. The results confirmed that HKUOT-S2 protein treatment significantly enhanced osteoblastic ALP activity compared to the osteoblasts control (Fig. 5G). Furthermore, both HKUOT-S2 protein and E2 treatments significantly increased osteoblast biomineralization compared to the osteoblast control (Fig. 5H). The HKUOT-S2 protein also significantly increased osteoblasts biomineralization compared to the E2 treatment group (Fig. 5H). These results were consistent with our previous findings which demonstrated that HKUOT-S2 protein could enter hMSCs to enhance hMSCs-osteoblasts differentiation [25]. The results demonstrated that HKUOT-S2 protein estrogenic activities induced robust enhancement of osteoblasts differentiation and functions. The current data suggests that HKUOT-S2 protein could be used as a positive ERs modulator to enhance osteogenesis.

3.6. Estrogen receptor (ER) antagonist reduced HKUOT-S2 protein-induced ERs upregulation

Estrogen receptor (ER) antagonists such as fulvestrant have a higher affinity for ERs than estrogen [73]. The competitive binding of fulvestrant to ERs leads to ERs inactivation and downregulation which in turn suppresses estrogenic signal transductions and associated biological activities [73,74]. Here, the ERs functions were inhibited with fulvestrant treatment to test if HKUOT-S2 protein-induced estrogenic activities in osteoblasts were mediated by the ERs. The hMSCs were differentiated into osteoblasts with fulvestrant treatment w/o E2 or HKUOT-S2 protein (Fig. 6A). Gene expression analysis by qPCR revealed that fulvestrant treatment reduced *ERα* and *GPR30* but not *ERβ* expressions when compared to the osteoblast control. HKUOT-S2 protein and E2 treatments restored the *ERα* and *GPR30* mRNA expressions to the level of the osteoblast control (Fig. 6B). Immunofluorescence staining results showed that fulvestrant treatment significantly reduced ERα, ERβ, and GPR30 protein expressions compared to the osteoblasts control. Fulvestrant treatment also significantly suppressed HKUOT-S2 protein and E2-induced upregulation of ERα, ERβ, and GPR30 protein expressions compared to the osteoblasts control (Fig. 6C–H). Western blot analyses also revealed that fulvestrant treatment suppressed ERα and GPR30 protein expressions in the osteoblasts (Fig. 6I). The results implied that HKUOT-S2 protein estrogenic activities were, indeed, mediated by ERs.

3.7. Fulvestrant treatment impaired HKUOT-S2 protein-induced osteoblasts differentiation and functions

Research studies have shown that fulvestrant treatment impaired BMP2-induced osteoblast differentiation and activities [75]. To test if fulvestrant inhibitory effects on ERs affected HKUOT-S2 protein-induced osteoblasts differentiation potentials, the fulvestrant-treated hMSCs-derived osteoblasts w/o E2 or HKUOT-S2 protein treatments were subjected to qPCR, immunofluorescence and Western blot analyses. The qPCR results showed that the fulvestrant inhibition of ERs impaired the HKUOT-S2 protein or E2 treatment-induced osteoblasts differentiation by reducing *ALP, COL1A1,* and *RUNX2* expressions when compared to the osteoblasts control (Fig. 7A). Both the immunofluorescence and Western blot results also showed that the downregulation of ERs by fulvestrant treatment corresponded to significant reduction of ALP protein expression when compared to that of the osteoblast control, E2, and HKUOT-S2 protein (Fig. 7B–F). The HKUOT-S2 protein treatment could significantly increase ALP protein expression with respect to the osteoblasts control under physiological conditions. However, fulvestrant treatment reduced the HKUOT-S2 protein-induced ALP protein expression to that of the osteoblasts control level (Fig. 7B–F). Furthermore, fulvestrant treatment significantly reduced RUNX2 protein expression when compared to the osteoblasts control (Fig. 7B–F). Unlike the ALP protein expression, the HKUOT-S2 protein or E2 treatment could not rescue the fulvestrant-suppressed RUNX2 protein expression (Fig. 7B–F). Next, we assessed whether the fulvestrant-impaired HKUOT-S2 protein-induced osteoblasts differentiation also affected the osteoblast functions. The fulvestrant-treated hMSCs-derived osteoblasts w/o E2 or HKUOT-S2 protein treatments were processed for ALP activity and Alizarin Red S (ARS) staining analyses. The results showed that fulvestrant treatment significantly reduced both the ALP activity and osteoblast biomineralization compared to the HKUOT-S2 protein, osteoblast, and E2 controls (Figs. S4A–D). There were no statistical differences in ALP activity and osteoblasts biomineralization among HKUOT-S2 protein, osteoblasts, and E2 controls (Figs. S4A–D). The HKUOT-S2 protein-induced ALP activity and biomineralization were usually higher than the osteoblasts control under normal circumstances without the influence of fulvestrant treatment. The result therefore implied that HKUOT-S2 protein treatment could partially rescue the fulvestrant-impaired ALP activity and osteoblasts biomineralization. This result was consistent with a study that reported that fulvestrant treatment significantly reduced BMP2-induced osteoblast mineralization [75]. Taken together, the data showed that ER antagonist treatment suppressed the estrogenic activities of HKUOT-S2 protein leading to the impairment of HKUOT-S2 protein-induced osteoblasts differentiation and biological activities.

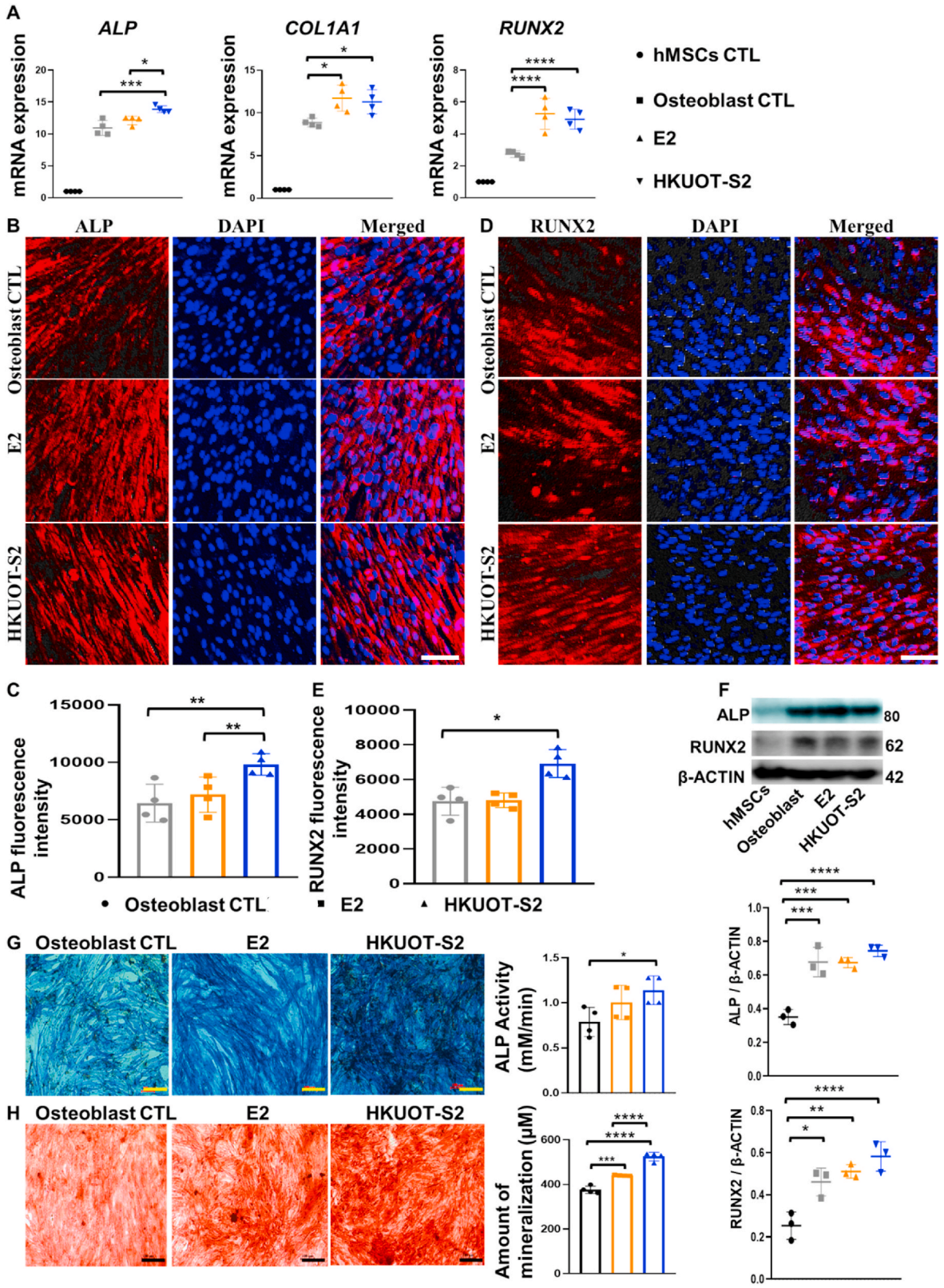


Fig. 5. HKUOT-S2 protein promoted osteoblasts differentiation and functions. A) HKUOT-S2 protein treatment increased osteogenic markers *ALP, COL1A1,* and *RUNX2* mRNA expressions in hMSCs-derived osteoblasts. B-E) Representative immunofluorescence staining images and quantification of ALP and RUNX2 protein expressions in osteoblasts. F) Representative Western blot images and quantification of ALP and RUNX2 protein expressions in osteoblasts. G) HKUOT-S2 protein treatment significantly enhanced osteoblastic ALP activity. H) HKUOT-S2 protein treatment significantly increased osteoblast biomineralization. n = 4, white scale bars = 50 μm, black and yellow scale bars = 100 μm. The values were shown as mean ± SEM, n = 4. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

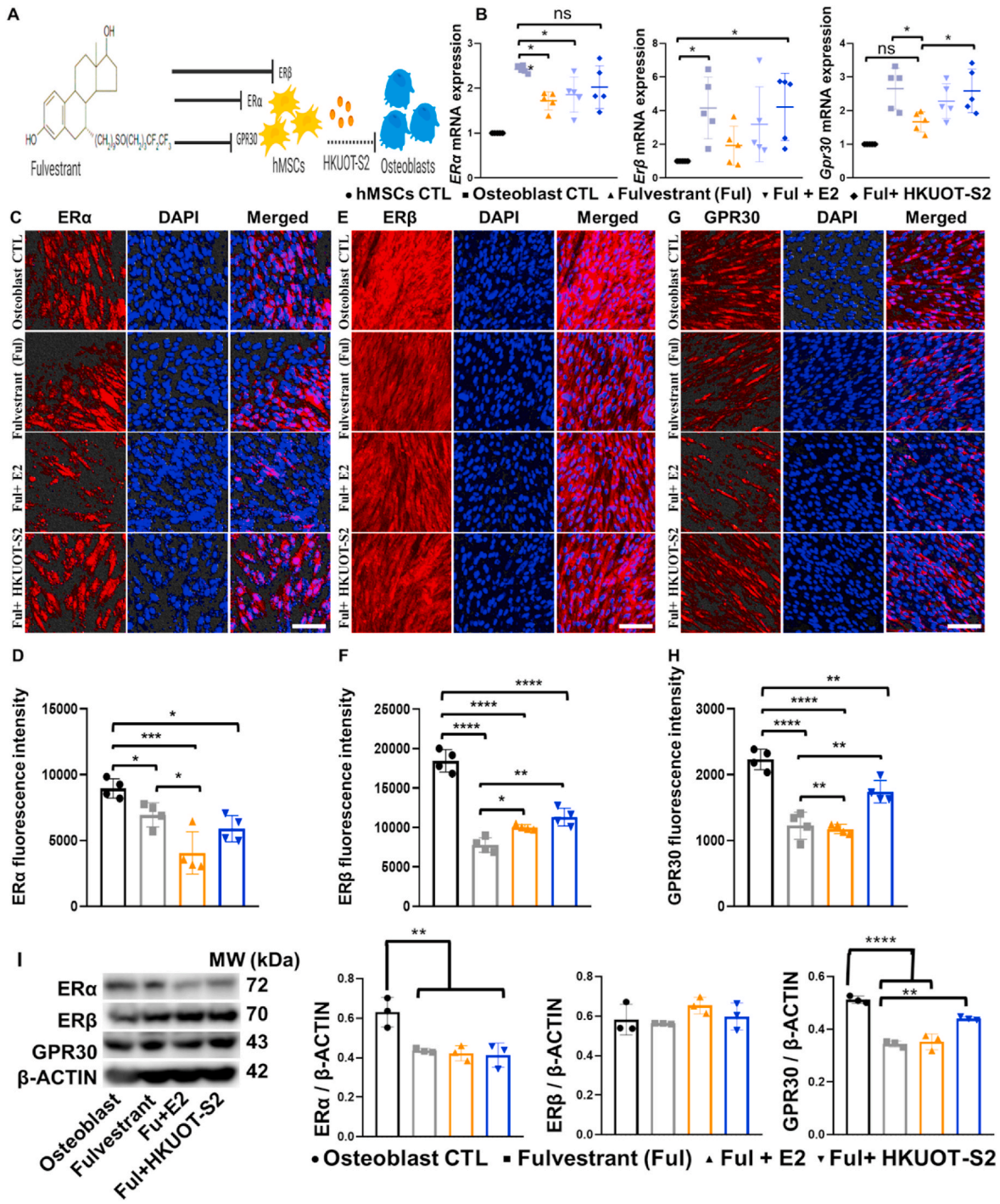


Fig. 6. Fulvestrant treatment suppressed estrogen receptor expressions in osteoblasts. A) Schematic diagram showing that fulvestrant treatment suppressed estrogen receptors ERα, ERβ, and GPR30 during hMSCs-osteoblasts differentiation. B) Effects of fulvestrant treatment on *ERα, ERβ*, and *GPR30* expressions during hMSCs-osteoblasts differentiation. (C–H) Representative immunofluorescence staining images and quantified fluorescence intensities of estrogen receptor ERα, ERβ, and GPR30 in fulvestrant treated osteoblasts. I) Western blot analysis of ERα, ERβ, and GPR30 protein expressions in fulvestrant-treated osteoblasts. The values were shown as mean ± SEM, n = 4, white scale bars = 50 μm, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3.8. HKUOT-S2 protein directly binds to estrogen receptors (ERs)

Both the *in vivo* and *in vitro* results consistently demonstrated that the HKUOT-S2 protein could increase the expressions of ERs to promote osteogenic functions. However, how the hKUOT-S2 protein interacted with the ERs was not clear. Immunoprecipitation (IP) analysis was therefore performed to establish possible HKUOT-S2 protein-ERs interaction using ERα, ERβ, GPR30, and IgG (control) antibodies (Fig. 8A). The HKUOT-S2 protein in SDS-PAGE was subjected to silver staining to confirm its molecular weight of 32 kDa (Fig. 8B). The IP analyses by Western blot and silver staining confirmed that the HKUOT-S2 protein was precipitated by ERα, ERβ and GPR30 antibodies in the IP elute and separated in the SDS-PAGE according to the molecular weight (32 kDa) (Fig. 8C). The HKUOT-S2 protein was not precipitated by the IgG antibody indicating the specificity of the HKUOT-S2 protein affinity to the ERs (Fig. 8C). The results indicated that the HKUOT-S2 protein

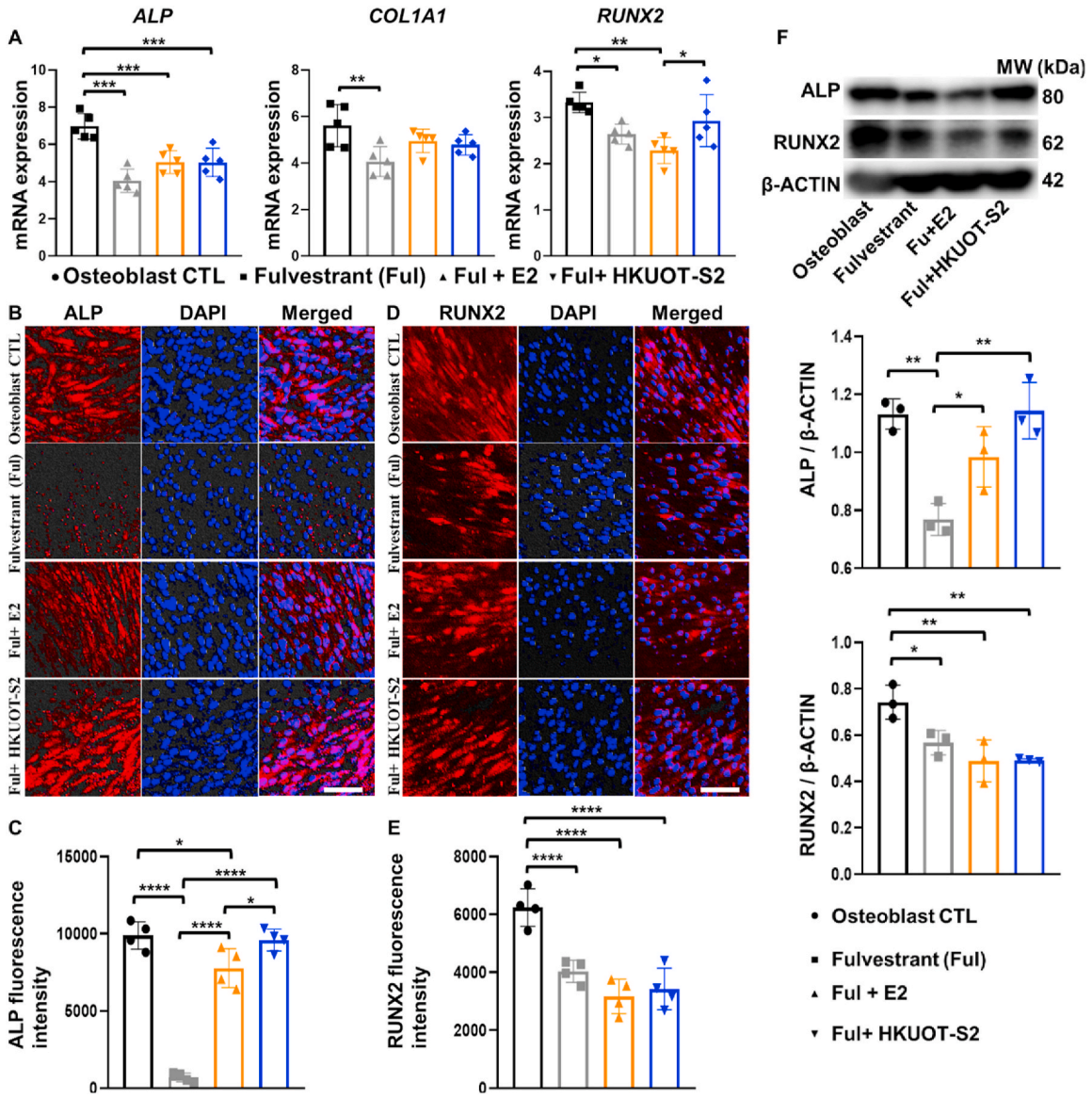


Fig. 7. Fulvestrant treatment suppressed HKUOT-S2 protein-induced osteoblasts differentiation. A) qPCR analyses of osteogenic gene expressions in fulvestrant treated osteoblasts. B-E) Representative immunofluorescence staining images and quantified fluorescence intensities of ALP and RUNX2 expressions in fulvestrant-treated osteoblasts. F) Representative Western blot images and quantifications of ALP and RUNX2 expressions relative to the housekeeping protein β-ACTIN during fulvestrant-treated hMSCs-osteoblasts differentiation. n = 4 for qPCR and immunofluorescence staining, n = 3 for Western blot, white scale bars = 50 μm. The values were shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

interacted with the ERs by binding to the ERα, ERβ and GPR30 antibodies to form HKUOT-S2 protein- ERs complexes which were eluted out from the agarose resin. The results implied that direct interaction between the HKUOT-S2 protein and the ERs might have contributed to the upregulation of the ERα, ERβ, and GPR30 expressions observed in both the *in vivo* and *in vitro* studies. The HKUOT-S2 protein could therefore bind to ERs and activate the ERs' activities to promote osteogenesis and maintain bone health.

3.9. Propose biological mechanisms by which HKUOT-S2 protein modulates ERs to suppress osteoporosis

The *in vitro* data of the current study suggested that the HKUOT-S2 protein could physically interact with the ERs and positively modulate osteoblastic ERα, ERβ, and GPR30 expressions to promote osteoblasts differentiation and functions. Inhibition of ERs with fulvestrant reduced the HKUOT-S2 protein-induced osteoblasts differentiation and functions (Fig. S5). It was therefore proposed that the HKUOT-S2 protein exerted anti-osteoporotic properties via skeletal estrogen receptor signaling to promote new bone formation, maintain bone microarchitecture, and prevent OVX-induced osteoporotic bone loss as illustrated by schematic diagram 1 (see Scheme 1).

4. Discussion

Clinical data have shown that osteoporotic bones deteriorate with high susceptibility to various fractures [76,77]. Estrogen deficiency is one of the most common etiologies of age-related osteoporosis predominant in postmenopausal women [1,7,78–81]. Different types of anti-osteoporotic drugs and therapies such as bisphosphonates, estrogen replacement therapy (ERT), and selective estrogen receptor modulators (SERMs) have been extensively used to improve the bone quality of osteoporotic patients [13,47–52]. However, there are major safety concerns about the potential side effects of these anti-osteoporotic

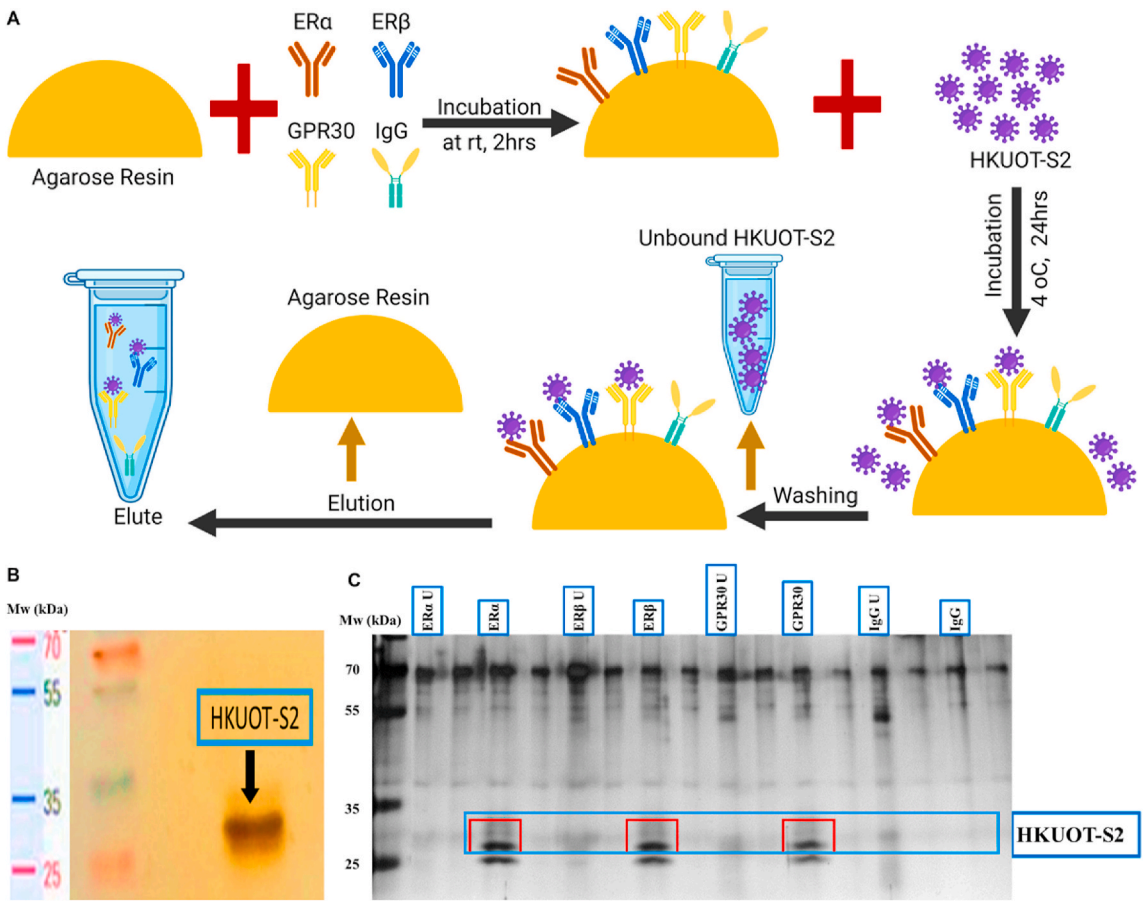


Fig. 8. HKUOT-S2 protein interacts with estrogen receptors (ERs. A) Immunoprecipitation (IP) showing the interaction between HKUOT-S2 protein and ERα, ERβ, and GPR30 antibodies. B) Western blot and silver staining analyses of HKUOT-S2 protein in SDS-PAGE. C) Western blot and silver staining analyses of the IP elute proteins separated in SDS-PAGE. ERα U= IP elute with HKUOT-S2 protein unbound to ERα antibody, ERα = IP elute with HKUOT-S2 protein bound to ERα, ERβ U= IP elute with HKUOT-S2 protein unbound to ERβ antibody, ERβ = IP elute with HKUOT-S2 protein bound to ERβ, GPR30 U= IP elute with HKUOT-S2 protein unbound to GPR30 antibody, GPR30= IP elute with HKUOT-S2 protein bound to GPR30, IgG U= IP elute with HKUOT-S2 protein unbound to IgG U antibody, IgG = IP elute with HKUOT-S2 protein bound to IgG.

treatment options. For instance, studies have shown that bisphosphonates can cause jawbone osteonecrosis, severe musculoskeletal pains, femoral cracks, and increase the risk of esophageal cancer development in osteoporotic patients [13,47,48]. Clinical studies have also reported that osteoporotic patients undergoing ERT treatment have high risks of developing breast cancers, and cardiovascular diseases [13,53,54]. SERMs treatment, deemed safer than ERT, has also been linked to thromboembolic disorders, muscle cramps, and stroke in osteoporotic patients [13]. The paramount goals of osteoporosis treatment protocols are to ensure that the ideal anti-osteoporotic interventions can suppress or prevent osteoporosis progression and maintain bone integrity close to the physiological conditions without compromising the general health of the patients. We have previously demonstrated via comprehensive *in vivo* toxicity studies that the newly discovered HKUOT-S2 protein was biocompatible with no toxic effects on the main body tissues such as the blood, heart, brain, liver, kidney, lungs, and spleen [25]. The low molecular weight HKUOT-S2 protein is therefore safe for *in vivo* applications such as osteoporosis treatment. The minimal safety concerns of the HKOUT-S2 protein may be explained by the report that low-molecular-weight proteins are biocompatible, bioavailable, and biodegradable but evoke less immunogenic responses [82].

We previously established an optimized effective dose of 0.1 μg/ml (3.125 nM) HKUOT-S2 protein treatment with robust osteogenic properties when compared to 0.1 μg/ml BMP-2 protein treatment *in vitro* [25]. Hence, the 3.125 nM HKUOT-S2 protein treatment was employed for osteoblast differentiation in the current *in vitro* studies. Studies have shown that 10–100 nM E2 treatments could enhance osteoblast differentiation *in vitro* [35,83]. It was shown in the current study that the low dose 3.125 nM HKUOT-S2 protein treatment also upregulated estrogen receptors (ERs) to enhance osteoblast differentiation and activity when compared to 10 nM E2 treatment *in vitro*. The current results indicated that the estrogenic HKUOT-S2 protein is potent and could be potentially applicable as an E2 replacement for promoting osteogenesis. Furthermore, the optimized effective dose of 2.18 mg/kg HKUOT-S2 protein treatment used to promote bone defect healing *in vivo* in the previous study [25] was also employed in the current osteoporosis suppression study. Indeed, consistent with the *in vitro* data, the 2.18 mg/kg HKUOT-S2 protein treatment also exhibited stronger estrogenic and osteogenic activities to maintain bone health under osteoporotic conditions *in vivo*. Both the *in vitro* and *in vivo* results collaboratively suggest that the biocompatible HKUOT-S2 protein could, indeed, be a suitable E2 replacement alternative for suppressing osteoporosis development.

Many animal studies have demonstrated that OVX can induce osteoporosis in the femur and lumbar vertebrae due to estrogen deficiency [63–67]. Indeed, our current study also confirmed that OVX induced femoral and vertebral osteoporosis in mice. It was reported that iron oxide nanoparticles with antioxidative properties could positively modulate bone metabolism to prevent OVX-induced osteoporosis development [7]. Another study also showed that a combination of plant-based quercetin (polyphenol) and BMP-2 protein treatment promoted osteogenesis and suppressed OVX-induced osteoporosis [6]. Our current data also demonstrated that the HKUOT-S2 protein can maintain

bone integrity and suppress osteoporosis development as that of the sham control and E2 treatment groups. It has been reported that estrogen treatment 5 times per week suppressed the OVX-induced femoral bone loss in mice [63]. We have demonstrated in the current study that 3 times per week HKUOT-S2 protein treatment prevented OVX-induced femoral and vertebral bone losses in mice. The current data revealed that there were no statistical differences between the HKUOT-S2 protein and E2 anti-osteoporotic properties. Clinical reports indicate that femoral and lumbar vertebral fractures are among the topmost osteoporosis-associated bone fractures [76,77]. The anti-osteoporotic potential of the HKUOT-S2 protein can therefore help to reduce the incidence of femoral and vertebral bone fractures in elderly osteoporotic patients. Consequently, the affordable and biocompatible HKUOT-S2 protein can be potentially applicable as an alternative anti-osteoporotic compound in place of ERT to minimize any potential side effects.

Different studies have shown that bone cells such as osteoblasts express estrogen receptors (ERs) ERα, ERβ, and GPR30 [15,42,84,85]. It has been well established that estrogen (E2) binds to the osteoblastic ERs to exert osteogenic and osteoprotective functions [15,85]. There are, however, limited data on the effects of osteoporosis on ERs expressions. One study reported that osteoporotic patient-derived primary mesenchymal stem cell (MSCs)-differentiated osteoblasts expressed decreased *ERα* and osteogenic marker, *ALP* [35]. The same study showed that E2-treated MSCs-derived osteoblast exhibited elevated *ERα* and *ALP* expressions [35]. Another study demonstrated that GPR30 activation by G1 (GPR30 agonist) treatment did not only promote neonate rat osteoblast differentiation but also suppressed OVX-induced bone loss in adult rats [32]. These data suggest that estrogen deficiency-induced osteoporosis could also decrease osteoblastic ERs expressions contributing to osteoporotic bone loss. Indeed, our current data showed that OVX-induced osteoporosis could be attributed to decreased skeletal ERα, ERβ, and GPR30 expressions as well as impaired osteoblast differentiation and functions. ERs such as ERα and GPR30 could therefore be therapeutic targets for osteoporosis treatment. It was demonstrated in the current study that the HKUOT-S2 protein treatment could prevent osteoporosis development by positively modulating osteoblastic ERs expressions and osteoblastic functions. Hence, this biocompatible and estrogenic HKUOT-S2 protein could serve as a new estrogen receptor modulator for suppressing osteoporosis progression.

Furthermore, there has been a significant decline in the clinical application of ERT for treating osteoporosis in menopausal women due to the ERT-associated side effects [53]. Consequently, current studies have focused on the application of selective estrogen receptor modulators (SERMs) to suppress osteoporosis [13,15,51,55,56]. The application of SERMs minimizes the clinical setbacks of ERT in osteoporotic patients. Indeed, the HKUOT-S2 protein used in the current study can also upregulate skeletal ERα and ERβ to maintain bone health and prevent OVX-induced osteoporosis progression. Our results were consistent with other studies which reported that SERMs such as raloxifene and bazedoxifene could activate skeletal ERα and ERβ expressions to prevent osteoporosis development [86–89]. Currently, raloxifene and bazedoxifene are the only FDA-approved SERMs for osteoporosis treatment in menopausal women [87,89]. Although the SERMs are considered clinically safer than ERT, both raloxifene and bazedoxifene treatments have been associated with adverse effects such as muscle cramps, stroke, and thromboembolic disorders in osteoporotic patients [13,88]. Discovering new SERMs with minimal clinical side effects will be very attractive to osteoporotic patients. We have shown that the HKUOT-S2 protein exerts estrogenic effects by positively upregulating skeletal ERs expressions to suppress both femoral and lumbar vertebral osteoporosis. Both animal and human studies have also demonstrated that daily administration of raloxifene or bazedoxifene prevented femoral and lumbar vertebral osteoporosis [88,90]. The HKUOT-S2 protein can be functionally considered as a new potential SERM for treating osteoporosis. The functions of SERMs have been largely limited to the modulation of the two classical ERα and ERβ. There is also limited data on the effects of SERMs on other ERs such as GPR30. The GPR30 is a novel transmembrane estrogen receptor that was recently discovered [91,92]. Although GPR30 expression in bone tissue has been reported, there is limited data on the GPR30 functions in the modulation of bone metabolism. The GPR30 protein could be one of the therapeutic targets for osteoporosis treatment. Indeed, the current results indicated that HKUOT-S2 protein-induced GPR30 upregulation might have also contributed to osteoporosis prevention. The application of HKUOT-S2 protein as an anti-osteoporotic SERM can offer some advantages to osteoporotic patients. Firstly, the HKUOT-S2 protein is a biocompatible phytoprotein isolated from edible plants. It is therefore highly anticipated that the HKUOT-S2 protein application will be clinically safer for osteoporotic patients. Secondly, we have demonstrated in animal studies that administration of HKUOT-S2 protein thrice per week was enough to suppress osteoporosis whereas daily treatments of raloxifene or bazedoxifene were required to achieve anti-osteoporotic effects in both animals and humans. The lesser frequency of HKUOT-S2 protein administration might be more convenient for osteoporotic patients in the future. Thirdly, in addition to the upregulation of ERα and ERβ proteins by raloxifene and bazedoxifene, the HKUOT-S2 protein also increased skeletal GPR30 protein to suppress osteoporosis. It is worth noting that bisphosphonates, estrogen, and raloxifene are anti-resorptives that suppress osteoclast activity to prevent osteoporosis. On the other hand, the HKUOT-S2 protein is an osteoanabolic phytoprotein that promotes osteoblast functions to suppress osteoporosis. There are always increasing demands for anti-osteoporotic drugs due to increasing global osteoporosis incidence. For instance, over 200 million osteoporotic patients with 8.9 million osteoporosis-associated bone fractures were estimated globally in 2020 [5]. Globally, osteoporosis-associated bone fractures have been projected to increase by 240 % and 310 % in women and men respectively by 2050 [80]. This implies that the discovery of a new anti-osteoporotic HKUOT-S2 protein can be helpful to millions of osteoporotic patients. The HKUOT-S2 protein could therefore be applied as an alternative, economical, biocompatible, and positive modulator of skeletal ERα, ERβ, and GPR30 to prevent osteoporosis.

The main limitation of the current study was the possibility that the HKUOT-S2 protein could diffuse into other tissues besides the target bone. Although, it was previously demonstrated that the therapeutic dose of the HKUOT-S2 protein treatment had no toxic effects on body tissues such as the blood, kidney, and liver *in vivo* [25], bone-specific HKUOT-S2 protein treatment for osteoporosis suppression would be more clinically attractive. As part of future research directions, the HKUOT-S2 protein can be loaded into bone-targeting nanocarrier delivery systems such bone targeting fluffy surface hybrid nano-adjuvant [58] to prevent osteoporosis development. We also plan to develop HKUOT-S2 protein-derived functional peptides with osteoanabolic properties for downstream osteoporosis treatment.

5. Conclusion

In conclusion, we have recently discovered a biocompatible HKUOT-S2 protein that can positively modulate osteoblastic and skeletal ERα, ERβ, and GPR30 functions to promote osteogenesis and new bone formation, maintain bone microarchitecture, and prevent osteoporosis. We have also demonstrated that estrogen receptor antagonist treatment can impair the estrogenic and osteogenic functions of the HKUOT-S2 protein. The current data therefore revealed that the HKUOT-S2 protein can promote osteogenesis to prevent osteoporosis via the estrogen receptor signaling pathway. Currently, only anti-resorptive SERMs are being used for treating osteoporosis. The HKUOT-S2 protein can serve as a new osteoanabolic agent with positive skeletal estrogen receptor modulatory properties for suppressing osteoporosis.

Statement of ethics

All the animal housing and experimental procedures were strictly performed by following the approved protocol by the HKU Ethics Committee, Committee on the Use of Live Animals in Teaching and Research (CULATR), (CULATR 5843–21).

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Data availability statement

All the research data are available in this article and the supplementary file.

Ethics approval and consent to participate

All the animal housing and experimental procedures were strictly performed by following the approved protocol by the HKU Ethics Committee, Committee on the Use of Live Animals in Teaching and Research (CULATR), (CULATR 5843–21).

CRediT authorship contribution statement

John Akrofi Kubi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Augustine Suurinobah Brah:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Kenneth Man Chee Cheung:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Andy Chun Hang Chen:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis. **Yin Lau Lee:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Kai-Fai Lee:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Wei Qiao:** Writing – review & editing, Writing – original draft, Validation, Formal analysis. **Yibin Feng:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Kelvin Wai Kwok Yeung:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

Kelvin Wai Kwok Yeung is an associate editor and Wei Qiao an editorial board member for Bioactive Materials and were not involved in the editorial review or the decision to publish this article. The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2024.08.045.

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