THE INVOLVEMENT OF NOVEL ESTROGEN RECEPTORS IN MEDIATING RAPID ESTROGENIC EFFECTS OF ER-XIAN DECOCTION AND ICARIIN IN BONE

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The Involvement of Novel Estrogen Receptors in Mediating Rapid Estrogenic Effects of Er-Xian Decoction and Icariin in Bone

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Certificate Originality

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Abstract

The estrogen-like bone protective effects of Er-xian decoction (EXD), a Traditional Chinese medicine (TCM) formula, and its abundant flavonoid, icariin, in postmenopausal women have been widely studied; such effects involve the activation of rapid estrogen signaling in osteoblast. Our previous study revealed no binding affinity of icariin to classical estrogen receptors α (ER-α or now called ER-α66) and the involvement of ligand-independent rapid estrogen signaling in mediating the actions of icariin. These suggested that ER-α66 might not be the sole estrogen receptors (ERs) responsible for the estrogenic actions of icariin and icariin-containing EXD. Recently, two novel membrane ERs, estrogen receptor α36 (ER-α36) and G-protein coupled estrogen receptor (GPER), were discovered and reported to mainly mediate rapid estrogen signaling through physical and functional interaction with ER-α66 in estrogen-sensitive tissues. Few studies also found their expressions correlated with bone remodelling. Thus, we hypothesized that ER-α36 and GPER might be the therapeutic targets of icariin and EXD to mediate rapid estrogenic effects through crosstalk with ER-α66 in osteoblasts.

In the present study, we first determined that ER-α36 and ER-α66 expression were differentially regulated while ER-α36 and GPER expression were similarly regulated by 17β-estradiol (E_2) and icariin in osteoblasts. Also, the regulation of ER-α36 expression by E_2 or icariin was GPER-dependent and ER-α66-independent. These results provided new insights regarding the regulation of GPER, ER-α36, and ER-α66 expression in osteoblasts and their differential responses to E_2 and icariin.

Next, we investigated the role of ER-α36 and GPER in the bone protection using transient transfection or GPER specific blockers. Results demonstrated the negative roles of ER-α36 and GPER in ER-α66-mediated estrogenic bone formation induced by icariin in preosteoblast and osteoblast. Such effects are perhaps via translocation from cytoplasm to cell membrane and regulation of ER-α66/ER-α36 and ER-α66/c-src complexes in ERK and Akt rapid estrogen signaling. These results not only indicated the differential activation of ERs by icariin and E_2 in bone, but also the negative regulation of bone formation by ER-α36 or GPER and signaling pathway aroused by icariin or E_2.

As for EXD study, the *in vivo* study confirmed our previous findings that EXD could exert estrogen-like bone protection without inducing uterotrophic effects in mature female Sprague Dawley OVX rats. EXD also appeared to regulate the ER-α36 or GPER protein expression in tibia heads of rats, indicating the recruitment of these novel ERs in bone by EXD. Our *in vitro* study futher studied the roles of ER-α36 or GPER in estrogen-like effect of EXD in bone.

Results clearly demonstrated that the expression of ER-α36, GPER and ER-α66 were differentially regulated in preosteoblast and osteoblast, suggesting the negative regulation of ER-α36 or GPER in bone formation and rapid estrogen signaling pathway in response to EXD. The last part of the present study focused on examining the involvements of ER-α36 and GPER in bone protection of EXD or icariin in female osteoblast-specific ER-α knockout mice. The study was designed to address the difficulties in distinguishing the role of different ER-α isoforms in mediating the rapid estrogen responses in normal osteoblastic cells. The results might indicate icariin and EXD could exert both ER-α66-dependent and ER-α66-independent bone protective effects in osteoblastic cells. Icariin and EXD might require ER-α66 in regulating E_2-senstive bone marker expression, but bypass ER-α66 in promoting cell proliferation of osteoblast cells. Specifically, the rapid estrogen signaling might be preferentially mediated by ER-α36 and GPER over ER-α66 in osteoblast upon treatment of icariin and postmenopausal level of estrogen, presumably as a negative feedback to the actions of ER-α66.

In summary, our study provided new insights to extend our understandings of the mechanisms behind the bone protection of icariin and EXD suggesting ER-α36 and GPER might be the target receptors in mediating rapid estrogen signaling.

List of publication

1. **Wong K-Y,** Zhou L-P, Wong M-S. Icariin, the most abundant flavonoid in *Herba Epimedii*, exerted osteoprotective effect without uterotrophic activity. 10th International Symposium on Nutritional Aspects of Osteoporosis. 2017. Hong Kong, Poster 21.
2. Zhou L-P, **Wong K-Y,** Yeung H-T, Dong X-L, Xiao H-H, Gong AGW, Tsim KWK, Wong M-S. Bone Protective Effects of Danggui Buxue Tang Alone and in Combination With Tamoxifen or Raloxifene *in vivo* and *in vitro*. Frontiers in Pharmacology. 2018;9.
3. **Wong K-Y,** Zhou L-P, Wong M-S. Involvement of GPER and ER-α36 in Estrogenic Bone Protection of Icariin *in vitro*. Novel Aspects of Bone Biology, Keystone Symposia on Molecular and Cellular Biology. 2018. Utah, USA, Poster 2022.
4. **Wong K-Y,** Zhou L-P, Wong M-S. Rhizoma Drynariae (RD) exerted osteoprotective effect without uterotrophic activity. 14th The International Postgraduate Symposium on Chinese Medicine. 2018. Hong Kong, Poster 17.
5. Zhou L-P, **Wong K-Y,** Cao S-S, Poon C-C, Yu W-X, Chan C-O, Mok D-K, Dong X-L, Xiao H-H, Wong M-S. Water Extract of Chinese Herb Herba Epimedii (Yin Yang Huo) Exerted Tissue Selective Estrogenic Effects on Bone in Mature Ovariectomized Rats. 14th The International Postgraduate Symposium on Chinese Medicine. 2018. Hong Kong, Poster 21.
6. Zhou L-P, **Wong K-Y,** Poon C-C, Yu W-X, Chan C-O, Mok D-K, Dong X-L, Xiao H-H, Wong M-S. EXD Chinese Herbal Formula Did Not Alter the Bone Protective Effects of SERMs in Mature Ovariectomized Rats. Annual Meeting of American Society for Bone and Mineral Research. 2018, Montreal, Canada, Poster SUN-1003.
7. Zhou L-P, Poon C-C, **Wong K-Y**, Cao S-S, Yu W-X, Dong X-L, Lee W-Y, Zhang Y, Wong M-S. Prenylflavonoid icariin induces ERE-independent estrogenic responses in a tissue-selective manner. Journal of the Endocrine Society. 2019;4.
8. Zhou L-P, Poon C-C, **Wong K-Y**, Cao S-S, Dong X-L, Zhang Y, Wong M-S. Icariin ameliorates estrogen-deficiency induced bone loss by enhancing IGF-I signaling via the crosstalk with non-genomic ERα signaling (under review)
9. **Wong K-Y**, Zhou L-P, Yu W-X, Poon C-C, Wong M-S. Bone protective herbal medicine Er-Xian decoction interacted with tamoxifen or raloxifene *in vivo* and *in vitro*.(submitted)
10. Zhou L-P, **Wong K-Y**, Yu W-X, Poon C-C, Wong M-S. Selective Estrogen Receptor Modulators-like activities of Herba Epimedii (HEP) extract and its interactions with SERMs on bone in vivo and in vitro. (submitted)
11. **Wong K-Y**, Zhou L-P, Poon C-C,Yu W-X, Wong M-S. Involvement of GPER and ER-α36 in Estrogenic Bone Protection of Icariin *in vitro*. (in preparation)

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Abbreviations

AF-1	Activation Factor 1
AF-2	Activation Factor 2
Akt	Protein Kinase B
ALP	Alkaline Phosphatase
BJT	*Radix Morindae*
BMD	Bone Mineral Density
BMP-2	Bone Morphogenetic Protein-2
BMSCs	Bone Mesenchymal Stem
BV/TV	Bone volume over total volume
CMV-36	Expression vector of ER-α36
CMV-GPER	Expression vector of GPER
CMV-HA	Control vector
CO_2	Carbon Dioxide
Conn.D	Connectivity Density
CTX	Collagen Type I C-Terminal Telopeptide
DBD	DNA binding domain
DG	*Angelica Sinensis*
DMEM	Dulbecco's Modified Eargle Medium
DMSO	Dimethyl Sulfoxide
DPD	Deoxypyridinoline
E_2	17β-estradiol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER-α	Estrogen Receptor Alpha
ER-α36	Estrogen Receptor Alpha 36
ER-α66	Estrogen Receptor Alpha 66
ER-α66-V	Expression vector of Estrogen Receptor Alpha 66
ER-β	Estrogen Receptor Beta
ERE	Estrogen Responsive Element
ERK	Extracellular signal-regulated Kinase
ERs	Estrogen Receptors
EXD	Er-xian Decoction

FBS	Fetal Bovine Serum
FDA	The Food and Drug Administration
FLL	*Ligustri Lucidi Fructus*
FSH	Follicle-Stimulating Hormone
GPCR	G-protein-coupled Receptor
GPER	G-Protein coupled Estrogen Receptor
H&E	Hematoxylin-Eosin
HB	*Cortex Phellodendri*
HEK293	Human Embryonic Kidney 293
HEP	*Herba epimedii*
HPG	Hypothalamic-Pituitary-Gonadal
HRT	Hormone Replacement Therapy
ICR	Imprinting Control Region
IGF-1R	Insulin-like Growth Factor 1 Receptor
IGF-1	Insulin-like Growth Factor 1
IL-1	Interleukin-1
LBD	Ligand Binding Domain
LC	Littermate Control
LH	Insulin-like Growth Factor 1
mRNA	Messenger RNA
MAPK	Mitogen-Activated Protein Kinase
OA	oleanolic acid
OC-Cre	Cre recombinase driven by the human osteocalcin promoter
OCN	Osteocalcin
OPG	Osteoprotegerin
OVX	Ovariectomy
PI3K	Phosphatidylinositide 3-kinases
PKA	Protein Kinase A
PKC	Protein Kinase C
pOC-ERαKO	Osteocalcin-drive Osteoblast-specific ERα Knockout
PVDF	Polyvinylidene Fluoride
RANKL	Receptor Activator Nuclear Factor Kappa-B Ligand
Runx2	Runt-related transcription factor 2

SD	Sprague Dawley
SERMs	Selective Estrogen Receptor Modulators
SMI	Structure Model Index
Tb.N	Trabecular Bone Number
Tb.Sp	Trabecular Bone Separation
Tb.Th	Trabecular Bone Thickness
TCM	Traditional Chinese Medicine
TRAP	RANKL-induced Tartrate-Resists Acid
UA	Ursolic Acid
VOI	Volume of Interest
WT	Wide-type
XM	*Curculigo rhizome*
ZM	*Rhizoma Anmarrhenae*
αMEM	Alpha Modified Eargle Medium

Chapter 1. Introduction

1.1 Osteoporosis

Osteoporosis is a disease caused by abnormal bone remodelling and characterized by microarchitectural deterioration that increases the risk of skeletal fragility (Leali et al., 2011). Proximal femur, vertebrae, and hip are the most common sites for recurrent osteoporotic fractures (Oden, McCloskey, Johansson, & Kanis, 2013). Osteoporosis imposes a huge financial burden to the health care system in the world. The Asian Federation of Osteoporosis Societies study estimated that the occurrence of osteoporotic hip fracture in Hong Kong might increase from 9,590 in 2018 to 27,468 in 2050 while the direct medical cost might dramatically increase from USD 84.7 million in 2018 to USD 242.6 million in 2050 (Cheung et al., 2018).

Osteoporosis can be classified to be primary or secondary, according to the National Institutes of Health. Primary osteoporosis is related to estrogen deficiency and aging; secondary osteoporosis could be caused by certain medical conditions and medications that can disrupt bone reformation. Among them, primary osteoporosis is the most frequent type of osteoporosis in women (Hellekson, 2002). Black and Rosen reviewed that 1.5 million fractures in the United States are associated with osteoporosis each year in which postmenopausal women are the vast majority (Black & Rosen, 2016). To diagnose osteoporosis, bone mineral density (BMD) assessed by X-ray or dual-energy absorptiometry has been utilized as a measurement of bone health. The risk of osteoporosis was shown to increase with the values of BMD T-scores below -2.5 (Sheu & Diamond, 2016).

1.1.1 Bone remodelling

Bone remodelling is an essential and lifelong physiological process that replaces mature or damaged bone with new bone in discrete sites of the skeleton in order to maintain bone health (McGowen, 2004; Seeman, 2009). Normal bone remodelling consists of four stages and is tightly regulated by osteoclast for resorption and osteoblasts derived from mesenchymal stem cells (MSCS) for bone formation (J.A & Kneissel, 2017 ; Owen & Reilly, 2018).

First, in the resorption phase (Figure 1.1), osteoclasts are recruited and moved to the surface of the old bones. They secrete digestive enzymes that dissolve the mineral content from the organic matrix of mature bone to generate an acidic microenvironment (Hadjidakis & Androulakis, 2006). In this phase, deoxypyridinoline (DPD) is released to urine, and collagen type IC-terminal telopeptide (CTX) is degraded (Kuo & Chen, 2017). The second phase is the reversal phase. Osteoclasts undergo apoptosis automatically or triggered by macrophage-like cells, while osteoblast precursors are derived from mesenchymal progenitors (Hadjidakis &

Androulakis, 2006). In the formation phase, osteoblasts undergo proliferation and differentiation and become mature osteoblasts. They cleave type I collagen to procollagen type 1 N propeptide (P1NP) and procollagen type 1 C propeptide (P1CP) to increase bone rigidity and alkaline phosphatase (ALP) forming osteoid, osteonectin, osteopontin (Hadjidakis & Androulakis, 2006; Kuo & Chen, 2017). The last phase is mineralization in which the matrix of osteoid is mineralized with calcium to generate new bone (Hadjidakis & Androulakis, 2006). To monitor the rate of bone turnover, many molecules being synthesized or broken down during bone remodelling are used as bone markers. P1CP, P1NP, and ALP released during bone formation could be the promising markers for bone formation, while DPD and CTX released during bone resorption could be the markers for bone resorption (Delmas, Eastell, Garnero, Seibel, & Stepan, 2000; Kuo & Chen, 2017). Besides the metabolism of old bone, symbolic molecular regulation for bone turnover has been widely studied for the past 20 years. Receptor activator of nuclear factor kappa-B ligand (RANKL)/RANK/ osteoprotegerin (OPG) system discovered in the mid-1990s to regulate bone remodelling in the molecular base (Figure 1.2) (Boyce & Xing, 2007; Yasuda et al., 1998). Both RANKL and OPG are synthesized in osteoblast under hormone stimulation, like, estrogen. Free RANKL binds to its receptors, RANK on the surface of osteoclasts which activates bone resorption. This metabolism could be blocked by the binding OPG with RANK preventing excess bone resorption. Also, many shreds of evidence support that the induction of RANKL transcription also induces the transcription of OPG (Kostenuik & Shalhoub, 2001). It further confirms that the homeostasis of bone remodelling is governed by RANK with a positive feedback of OPG.

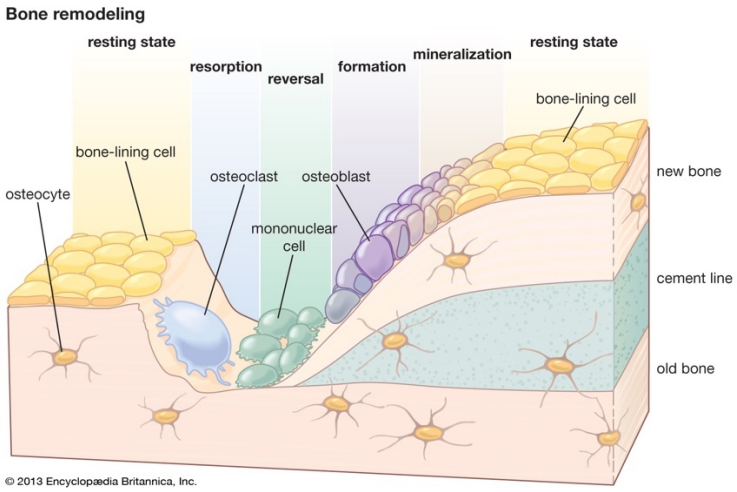


Figure 1.1 The process of bone remodelling. Bone remodelling is a process to replace old lining cells with new osteoid with the aid of osteoblast and osteoclasts.

There are four main stages in bone remodelling. Osteoclasts resorb the old bone lining cells upon activation of hormone in the resorption stage which followed by the recruitment of osteoblast precursors by unclassified macrophage-like cells in the reversal phase. Osteoblast precursors then differentiate and proliferate into mature osteoblasts with the release of ALP. The bone matrix is subsequently formed in the formation phase by mature osteoblast. Type one collagens are broken down to form P1CP and N1CP. Bone lining cells are then mineralized during mineralization. (Adapted from Encyclopaedia Britannica, 2013)

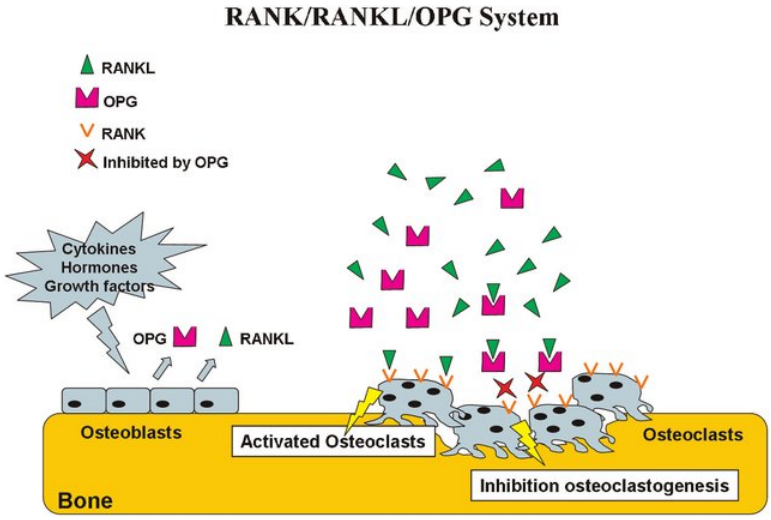


Figure 1.2 RANK/RANKL/OPG system.

Upon activation by cytokines, hormone or growth factors, osteoblasts release OPG and RANKL. The binding of RANKL to RANK on osteoclasts could promote osteoclastogenesis. The binding of OPG could inhibit this reaction to RANK in osteoclasts. Therefore, less free RANKL could activate osteoclasts. (Adapted from Nardone et al., 2014)

1.1.2 **Postmenopausal osteoporosis**

Postmenopausal osteoporosis is the most common type of osteoporosis in women at the age of 50 or higher, in which estrogen deficiency induced by ovarian depletion results in an increase in bone resorption without a corresponding escalation of bone formation. It leads to uncoupled bone remodelling and a net loss of bone (Dobbs, Buckwalter, & Saltzman, 1999; Watts, 2018). Studies found that the urinary DPD levels in normal women is lower than that in postmenopausal women while osteoporotic postmenopausal women have even higher urinary DPD level compared to postmenopausal control group on average (Bartram et al., 2006; Yilmaz, Bayram, Erbagci, & Kilincer, 1999). The discernible boost of DPD in urine is compelling proof of increased bone degradation in postmenopausal women upon estrogen deficiency. With respect to bone density in figure 1.3, it illustrates that BMD decreases significantly after the age of 60 in women. MCNabb reported that the mean changes of BMD are -3.6% at the total hip, -1.7% at the distal femur, and -1.3% at the lumbar spine in the first five years of menopause in women (McNabb et al., 2013). These results indicate the high risk of osteoporosis in postmenopausal women.

The lack of estrogen causes postmenopausal osteoporosis presumably via OPG/RANKL system. The low level of estrogen fails to stimulate the OPG synthesis from osteoblasts (Jia, Zhou, Zeng, & Feng, 2017). Less OPG is secreted to block RANKL from binding to RANK in osteoclast. In contrast, the production of the cytokines in osteoclastogenesis increases the secretion of RANKL, resulting in the promotion of early osteoclast differentiation progenitors to active osteoclasts by forming a complex with RANK (Figure 1.4). Subsequently, bone resorption overwhelms bone formation and lead to loss of bone mass when estrogen is inadequate (Michael, Harkonen, Vaananen, & Hentunen, 2005; Silva & Branco, 2011).

Extensive studies have proven that binding of estrogen with ERs on bone cells regulates OPG/RANKL system through complex pathways. In rapid estrogenic signaling, estrogen could bind to ER-α to activate Mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling pathway downregulating cytokines and growth factors such as interleukin-1 (IL-1), IL-1 which consecutively inhibits RANK/RANKL binding, curbs osteoclast activities and promotes its apoptosis (Shevde, Bendixen, Dienger, & Pike, 2000). Estrogen additionally takes part in gene regulation of retinoblastoma-binding protein 1, a Runt-related transcription factor 2 (Runx2) co-activator, and ALP. It consecutively activates Runx2 action to induce the differentiation of mesenchymal stromal cells (MSCs) to immature osteoblasts directing bone formation (Amzaleg et al., 2018).

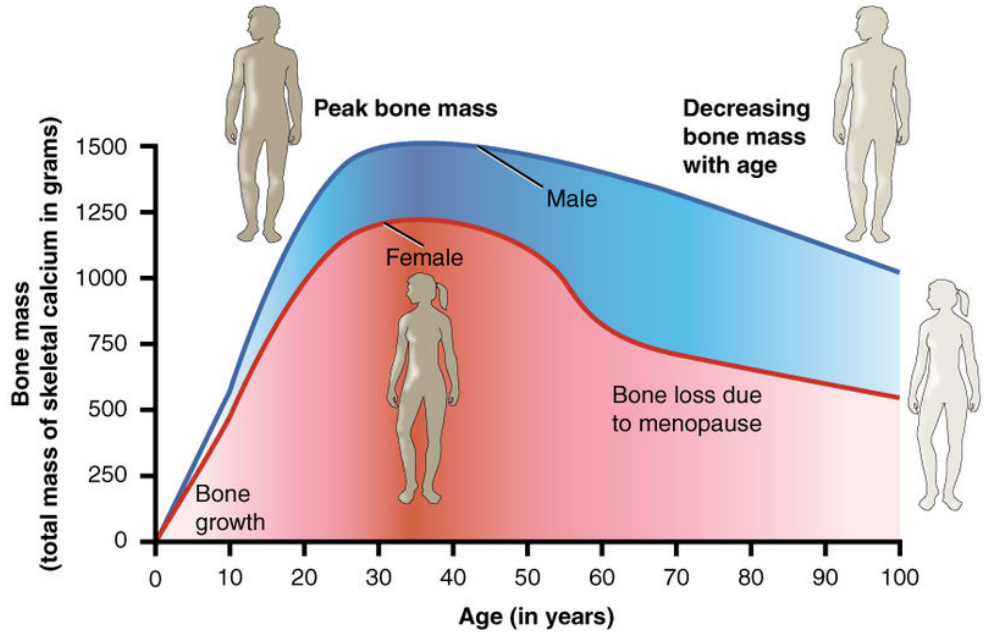


Figure 1.3 Bone mass changes with ages in male and female.

Bone mass increases continuously after birth until the age of 30 and 40 in male and female, respectively. Upon reaching the peak of bone mass, the decline of bone mass is more significant in the female than in male. The fracture threshold is around the age of 60 in women. (Adapted from Exercise, Nutrition, Hormones, and Bone Tissue, 2013)

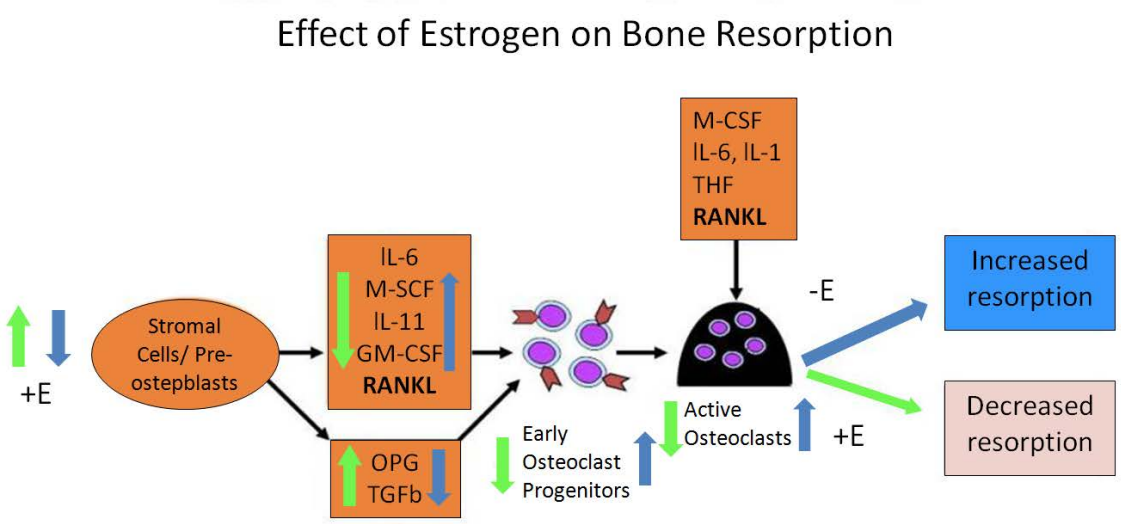


Figure 1.4 The effect of estrogen on bone resorption.

Estrogen could work on pre-osteoblast and monocytes. The decrease in estrogen (blue arrow) could decrease the release of OPG but increase the release of RANKL and interleukin-6 (IL-6). These may result in the development of active osteoclasts from early osteoclast progenitors. Bone resorption is then increased.

1.2 Overview of the current therapy for postmenopausal osteoporosis

Current therapy for postmenopausal osteoporosis could be classified into two groups: non-pharmacological interventions and pharmacological management. The mechanisms of improving bone health are either through the regulation of calcium content, osteoblastogenesis, osteoclastogenesis, adipogenesis, as well as the recently discovered microbiome (Eastell et al., 2019; Tella & Gallagher, 2014).

1.2.1 Non-pharmacological interventions

In terms of nutrition, a daily intake of 1200mg of calcium is recommended for osteoporotic women (Bolland et al., 2010). Calcium supplementation is recommended to compensate for the inadequate amount of dietary calcium. Moreover, an oral intake of 800IU of vitamin D3 per day, maintaining serum 25-hydroxyvitamin D level higher than 20 ng per mL, could also help to prevent deterioration of bone in women (Heaney et al., 2002). On the other hand, a healthy lifestyle could also promote bone formation and prevent bone loss, for instance, exercise and prevent smoking and drinking alcohol (Tella & Gallagher, 2014). However, improper consumption of calcium and vitamin D may cause constipation, kidney stone formation and myocardial infarction.

1.2.2 Pharmacological management

1.2.2.1 Drugs targeting bone remodelling

Bone remodelling is a lifelong process of bone breakdown by osteoclast followed by formation of new bone by osteoblasts. Antiresorptive drugs address the restoration of bone via reducing the rate of bone resorption and bone turnover mediated by osteoclast. Bone-anabolic drugs address the formation of bone by improving the rate of bone formation by osteoblast (Tella & Gallagher, 2014).

Bisphosphonate, one of the current the Food and Drug Administration (FDA) approved antiresorptive drugs, prevents osteoclastogenesis by inhibiting farnesyl diphosphate synthase (FDPS) in the mevalonate pathway (Khosla et al., 2007). It could interrupt osteoclast activity and survival through the prevention trafficking of signaling molecules, like Ras, Rac, Rho, and Rab in mevalonate pathway (Dunford, Rogers, Ebetino, Phipps, & Coxon, 2006; Gong, Altman, & Klein, 2011). Moreover, cathepsin-K inhibitors, an antiresorptive drug, could reduce matrix dissolution and bone resorption preventing type 1 collagen organic bone from degradation (Bone et al., 2010). However, administration of bisphosphonate is associated with atypical subtrochanteric fractures, osteonecrosis of the jaw, while the unpredictable off-target effect

was found in cathepsin K inhibitor treatment (Bone et al., 2010; Gong et al., 2011). In addition to western drugs, Traditional Chinese medicine (TCM) also regulates bone remodelling via suppression of osteoclastogenesis. *Ligustri Lucidi Fructus* (FLL) (Y. Zhang et al., 2006) and its active components, oleanolic acid (OA) and ursolic acid (UA) (Sisi Cao et al., 2018; S. Cao et al., 2018), were found to reduce RANKL-induced tartrate-resists acid (TRAP) activity and multinucleated osteoclast formation. Danshen, another well-known TCM for improving blood circulation, was reported to inhibit osteoclast activity and simulate osteoblastic formation by targeting RANKL/OPG pathway (Guo et al., 2014; H. Liu et al., 2018).

On the other hand, anti-Wnt inhibitor and teriparatide are the two common types of bone-anabolic drugs. Defective Wnt signaling pathway leads to impaired bone which could be prevented by inhibiting Wnt antagonist. The secreted frizzled-related proteins (sclerostin) and Wnt inhibitor factor (cerberus and Dkks) are the common groups of Wnt antagoinst (Neer et al., 2001). As regards teriparatide, it has a partially identical sequence of human parathyroid hormone which could increase the proliferation and bone formation processes in osteoblast (Brixen, Christensen, Ejersted, & Langdahl, 2004). However, reported side effects of teriparatide include lack of energy, pounding heartbeats, and constipation (Tella & Gallagher, 2014).

1.2.2.2 **Drugs targeting adipogenesis**

Mesenchymal stem cells (MSCs) can differentiate into several cell types. The commitment of MSCs into adipocytes is called adipogenesis, while differentiation of MSCs into osteoblast is called osteoblastogenesis. The shift of balance between these two processes determines the rate of bone formation (L. Hu et al., 2018). The third generation of bisphosphonate, Risedronate, was found to inhibit adipogenic differentiation, but promote osteoblastic differentiation of human MSCs via downregulation of RANKL (Neer et al., 2001). Also, a primary polyphenol in olive oil, oleuropein, was demonstrated to suppress adipogenesis via the reduction of adipogenic genes, including lipoprotein lipase and peroxisome proliferator-activated receptor-gamma in human MSCs(Brusotti et al., 2017; Santiago-Mora, Casado-Diaz, De Castro, & Quesada-Gomez, 2011). Moreover, betulinic acid was reported to promote the differentiation of murine preosteoblastic MC3T3-E1 cells but suppress differentiation markers in 3T3-L1 adipocytes(Brusotti et al., 2017).

1.2.2.3 **Drugs targeting calcium content**

Postmenopausal osteoporosis could be caused by low intestinal calcium absorption and low 1,25 dihydroxyvitamin D3 level (Santiago-Mora et al., 2011). In addition to increasing daily uptake of calcium, the administration of TCM could increase calcium content. FLL (Y. Zhang et al., 2006) and its activate component OA and UA (Sisi Cao et al., 2018; S. Cao et al., 2018) was found to suppress urinary calcium excretion and increase intestinal calcium absorption rate, bone calcium content as well as calcium utilization *in vivo* model, indicating their abilities to regulate calcium balance. Furthermore, Gushukang, a TCM formula for treating osteoporosis, was shown to maintain calcium homeostasis via regulation of vitamin D metabolism. Increase in calcium absorption in duodenum and decrease in urinary calcium excretion in ovariectomized mice treated with Gushukang affirmed its ability to improve bone health (X. L. Li, Wang, Bi, Chen, & Zhang, 2019).

1.3 Estrogen receptor-related therapy for postmenopausal osteoporosis

The primary cause of postmenopausal osteoporosis was due to inadequate estrogen production in ovary. Thus, targeting estrogen and its estrogen receptors (ERs) are the strategies of several treatments, such as hormone replacement therapy, selective estrogen receptor modulator, phytoestrogen as well as TCM formulas (Tella & Gallagher, 2014; Z.-Q. Wang et al., 2013).

1.3.1 **Hormone Replacement Therapy (HRT)**

HRT, also called menopausal hormone therapy, is previously considered as the gold standard method for relieving menopausal symptoms. HRT restores the estrogen deficiency by supplementing estrogen with or without progestin (Ji & Yu, 2015; Jyotsna, 2013). The Women Health Initiatives (WHI) reported a clinical trial in the United States to study the bone protective effects of HRT in 16,608 women in 2003. Figure 1.5 shown a remarkable decrease in hip fracture by 30-50% and all fractures by 20% in women after 2-year treatments with 0.625 mg/d conjugated equine estrogen when compared to women with placebo (Hulley & Grady, 2004). Unfortunately, severe side effects of HRT, including the risk of heart attacks, breast cancer, venous thromboembolism, endometrial cancer, pulmonary embolism, and colorectal cancer, were reported (Marco Gambacciani & Levancini, 2014; M. Gambacciani & Vacca, 2004).

1.3.2 **Selective estrogen receptor modulators (SERMs)**

SERMs are the western prescription drugs used to manage menopausal symptoms. They are ERs ligands that trigger anti-estrogenic or estrogenic effects. Tamoxifen, raloxifene, bazodoxifene, and lasofoxifene are common SERMs used for treatment of postmenopausal

syndrome (Komm & Chines, 2012; Martinkovich, Shah, Planey, & Arnott, 2014; Maximov, Lee, & Jordan, 2013). Their structures are similar to estradiol (Figure 1.6) in which the phenolic rings are responsible for ERs binding. Previous study revealed that the phenolic ring of E_2 could bind to Glu 353, Arg394, His 524 or Leu384 and Met421 in the ligand binding domain oc ERs. While the functional groups in-branch arouse different binding sites on ERs and the selectivity of SERMs at AF-2 domain in ERs. For tamoxifen and raloxifene, the bulky side chains could block the AF-2 domain by interrupting the protein structure at helix 12 in the ligand binding domain of ERs (Martinkovich et al., 2014).

Tamoxifen, the first-generation of SERMs, is clinically approved for the treatment of breast cancer. However, the beneficial side effect of tamoxifen on bone is observed. BMD measured in 179 postmenopausal women treated with tamoxifen were significantly increased by 1.17% in the spine, 1.71% in hip compared to those treated with placebo. Raloxifene is a second-generation of SERMs that is approved by FDA for the prevention and treatment of postmenopausal osteoporosis (Maximov et al., 2013). Multiple studies revealed that raloxifene treatment elevated the estrogenic effect to inhibit osteoclast differentiation and bone resorption (Gianni et al., 2004). Reports demonstrated that raloxifene has a robust anti-estrogenic effect in ovariectomized (OVX) rat models (Cranney et al., 2002). The risks of vertebral fracture were reduced by 30% in postmenopausal women receiving 60mg/d raloxifene (Jolly et al., 2003). Bazodoxifene is the third generation of SERMs that is found to prevent both vertebral and non-vertebral fractures in high-risk women with positive effects on lipid profile (Lindsay, Gallagher, Kagan, Pickar, & Constantine, 2009). Lasofoxifene is also the third generation of SERMs that has affinities for both ERα and ER-β similar to estradiol and about 10-fold higher than raloxifene and tamoxifen. This property contribute to the beneficial effect of laxofoxifene on bone health by reducing bone turnover, and preventing bone loss without stimulating breast diseases (Cummings et al., 2010).

Unfortunately, the use of SERMs were associated with severe side effects in a way similar to HRT. Tamoxifen was found to induce endometrial cancer and blood clots; while raloxifene was found to increase the risk of thromboembolic events in postmenopausal women (Komm & Chines, 2012; Martinkovich et al., 2014; Maximov et al., 2013). Bazedoxifene was reported to cause heart attack, stroke, cancer of breast, uterus, and ovary while lasofoxifene was found to increase the risk of venous thromboembolism and deep vein thrombosis (Gennari, Merlotti, De Paola, Martini, & Nuti, 2008). Thus, alternative approaches for the management of menopausal symptoms and estrogen-related diseases in postmenopausal women are needed.

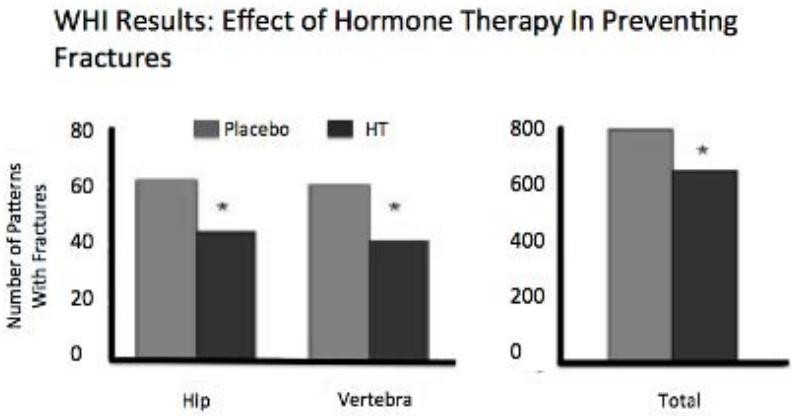


Figure 1.5 Effects of HRT in preventing fractures.

Numbers of fracture at the hip, lumbar spine, and total number (lower arm, wrist, and non-vertebral) of fracture in women significantly decreased upon HRT as compared to placebo. (Adapted from Hulley and Grady, 2004)

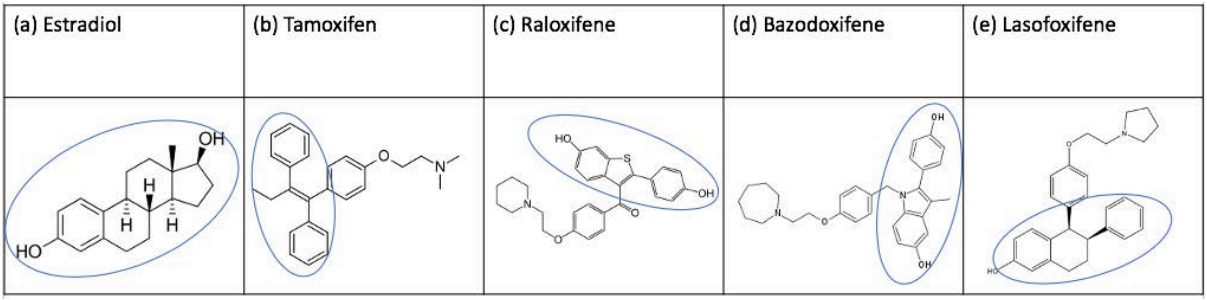


Figure 1.6 Structure of 17β-estradiol (E_2) and SERMs.

E_2 (a), tamoxifen (b), raloxifene (c), bazodoxifene (d), and lasofoxifene (e) contain phenolic ring (shown with blue circle) which is indispensable for binding to estrogen receptors. The functional groups of tamoxifen, raloxifene, bazodoxifene and lasofoxiene are responsible for the selective action on estrogen receptors as antagonists or agonists. (Adapted from Peterson, 2011).

1.3.3 **Chinese herbs and natural product**

According to the theory of Chinese Medicine, "kidney" is claimed to be the "prenatal basic of life" in Huang-Di-Nei-Jin, a famous monograph on traditional Chinese medicine. According to Nei-jin, the development and function of bone depend on "kidney essence" that contains "kidney yin" and "kidney yang." The balance between "Yi" and "Yang" is crucial to modulate tender and bone strength. Also, "essence" can transform into "bone marrow" to nourish bone, promote bone growth, repair and strengthen the skeleton. When "kidney essence" is inadequate, the bone formation is affected and leads to flaccidity of skeleton and hypoevolutism, brittleness of the bone and susceptibility to fracture in aged people (Fung & Linn, 2017).

In Chinese Medicine, there is no corresponding term to “osteoporosis”. However, in the view of pathogenesis and clinical manifestations, “osteoporosis” in modern science is mostly similar to “atrophic debility of the bone” in Chinese Medicine. Shen Ziyin first fused the concept of TCM with modern science and proposed that aging in the hypothalamic-pituitary-gonadal (HPG) axis was resulted from kidney-yang deficiency (S. J. Wang et al., 2016; ZY, 1995). Studies shown that "Kidney-tonifying" Chinese medicine could increase the estrogen level in rats with kidney deficiency induced by hydrocortisone and postmenopausal women upon treatment for six months (Y. Zhang et al., 2019). Since HPG axis are endocrine glands governing the production of the sex hormone, namely, estrogen. Thus, kidney deficiency is probably a joint pathological basis of estrogen deficiency and the development of osteoporosis because it could improve the circulating estrogen and promote bone growth.

As the bone loss is considered closely related to “kidney deficiency”, “kidney-tonifying” herbs have been utilized to treat osteoporosis since the ancient time. Commonly used “kidney-tonifying” TCM include *Curculigo rhizome (Y. Liu, Liu, & Xia, 2014)*, *Morinda*, *Herba epimedii* (R. H. Liu et al., 2015), *Rhizoma Drynaria* (R. W. K. Wong, Rabie, Bendeus, & Hägg, 2007) boosting “kidney yang” while *Angelica Sinensis* (Lim & Kim, 2014)*, Fructus Ligustri Lucidi* (Y. Zhang et al., 2006) enrich “kidney yin”. The action of enriching “kidney yin” and “kidney yang” by kidney-tonifying herbs could contribute to the bone development governed by “kidney essence”.

1.3.3.1 **Er-xian decoction(EXD)**

"Kidney-tonifying" Er-xian decoction (EXD) was designed by Zhang Bo-na, professor at the Shanghai University of Traditional Chinese Medicine in China in the 1950s and covered in a book "formula study of Chinese medicine" in China as a contemporary formula (Zhu, Li, Jin, Fang, & Zhang, 2014). It is a widely used Chinese Medicine to treat menopausal syndrome, osteoporosis, and age-associated disease in the past 60 years (H. Y. Chen, Cho, Sze, & Tong, 2008; L. L. Zhong et al., 2013). The name "Er-xian" means the two abundant and principal drug in this combination. They are *Herba epimedii* (HEP) and *Curculigo rhizome* (XM), which exert the most excellent effects among the rest of the herbs. Beside HEP and XM, EXD consists of four other herbs, including *Radix Morindae* (BJT), *Rhizoma Anmarrhenae* (ZM), *Cortex Phellodendri* (HB) and *Angelica Sinensis* (DG) (Lindsay et al., 2009). Each herb is formulated with a specific constitutional ratio and exerts distinct action (Table 1) (Zhu et al., 2014).

1.3.3.1.1 Bone protective effects of EXD

EXD was reported to exert estrogen-like anti-osteoporotic effect in clinical and preclinical studies. Bioactivity-guided fractionation has isolated 12 active anti-osteoporotic constituents from EXD, including icariin, anemarsaponin BII and berberine (Qin et al., 2008). Eight clinical trials revealed that 3-6-month EXD treatment increased serum calcium level for bone mineralization, ALP for bone differentiation and osteocalcin (OCN) for bone mineral metabolism in menopausal women at the age from 45 to 54 (J.-Y. Li et al., 2017) without causing adverse events in the reproductive system and other postmenopausal syndromes (Y. Wang, Lou, Shi, Tong, & Zheng, 2019). Also, EXD was shown to phenotypically improve BMD in femoral great trochanter, femoral neck, and lumbar spine in postmenopausal women (J.-Y. Li et al., 2017).

Preclinical studies showed that EXD displayed inhibitory effects on osteoclastogenesis by inhibiting the formation of bone resorption pits and several multinucleated osteoclasts in female Imprinting Control Region (ICR) mice (J.-Y. Li et al., 2017). On the other hand, EXD was also reported to improve static and dynamic histomorphometric parameters and biomechanical strength, such as bending, tensile, and compressive mechanics of the bones in OVX SD rats (Lim & Kim, 2014). In *ex vivo* study, EXD stimulated osteoblastic differentiation, self-renewal, but inhibit adipogenesis in bone mesenchymal stem (BMSCs) (S. Liu et al., 2016). Our previous studies shown that consecutive administration of EXD for 12 weeks could prevent OVX-induced bone loss at the trabecular bone in the distal femur and proximal tibia

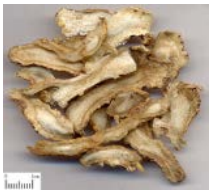
in four-month-old SD rats and stimulated the cell proliferation of rat osteoblast-like UMR-106 cells (K. C. Wong et al., 2014).

Notably, EXD could restore serum estradiol levels in both postmenopausal women as well as animal models. EXD could stimulate the production of ovarian estradiol by ovarian aromatase, and expression of ER-β (Sze et al., 2009). These, in return, regulate follicle-stimulating hormone (FSH) receptor-cyclic adenosine monophosphate (cAMP) pathways to phosphorylate protein kinase B (Akt) (Gonzalez-Robayna, Falender, Ochsner, Firestone, & Richards, 2000). Despite the upregulation of serum estradiol, no severe side effects on reproductive organs nor estrogen-sensitive tissues were observed in postmenopausal women as well as animal models upon treatment with EXD (Y. Wang et al., 2019). These findings suggest that EXD is a potential alternative approach to tissue-selectively manage postmenopausal osteoporosis.

1.3.3.1.2 Activation of estrogen signaling by EXD

Due to the complexity and the interaction of the active components in EXD, multiple mechanisms behind its bone protection were revealed. Networking pharmacology (S. Wang et al., 2015) and metabolism studies (Xue et al., 2011) revealed that the bone protection of EXD might arise through the regulation of lipid and energy metabolism, oxidation system, calcium, steroidogenesis as well as estrogen-related pathways. Liu's research group has revealed that EXD could alter 389 genes in OVX rats and EXD-treated BMSCs using Oligo GEArray Experiments. These included estrogen signaling pathways, such as MAPK signaling pathway (S. Liu et al., 2016). Our group has shown that EXD promoted the cell proliferation of UMR-106 cells that could be abolished by ICI 182,780, a specific ER inhibitor, suggesting ERs-mediated effects of EXD. In genomic signaling, EXD could activate ER-α and ER-β mediated ERE-dependent transcription in UMR-106 cells which indicates that EXD did not have selectivity toward ER-α nor ER-β (K. C. Wong et al., 2014). While in rapid estrogenic signaling, EXD induced phosphorylation of ER-α (Ser118) in UMR-106 cells and decreased the formation and activity of osteoclasts (K. C. Wong et al., 2014). However, the complex mechanisms behind the estrogenic bone protection of EXD are far from clear. The fundamental study is needed to understand the molecular regulation in estrogenic signaling by EXD in bone.

Table 1.1 Composition of EXD

Herb		Weight ratio	function of herb
Herba epimedii (Yinyanghuo,淫羊藿)		9	to tonify kidney yang, strengthen bones and muscles
Rhizoma curculiginl (Xianmao,仙茅)		9	to tonify kidney yang, strengthen bones and muscles
Radix Morindae (Bajitian,巴戟天)		9	to invigorate kidney yang, strengthen bones and tendons
Angelicae Sinensis (Danggui,當歸)		6	to nourish and active blood, regulate menstruation
Cortex Phellodendri (Huangbo,黃柏)		6	to clear heat, reduce fire and preserve yin
Rhizoma Anemarrhenae (Zhimu,知母)		9	to nourish yin, clear deficient fire and quenches thirst

1.3.4 **Phytoestrogen- icariin**

Phytoestrogen is a plant-derived non-steroidal compound that could functionally imitating human estrogen. (Kurzer & Xu, 1997). Flavonoid, the largest group of phytoestrogen, contains flavones, coumestans, and prenylflavonoids. The phenolic ring in flavonoid is indispensable for binding to ERs by mimicking estrogen. (Křížová, Dadáková, Kašparovská, & Kašparovský, 2019; Kurzer & Xu, 1997).

Icariin, a C-8 prenylated flavanol glucoside (Figure 1.7), is a major flavonoid compound in *Herba Epimedii*, the principal drug of EXD (M. S. Wong & Zhang, 2013). Icariin was found to be the active anti-osteoporotic constituent in EXD (Qin et al., 2008) and played a significant role in contributing to the estrogenic action of EXD. Due to the estrogenic effect of icariin, icariin is commonly promoted as a treatment for estrogen-related cardiovascular disease, osteoporosis, endometrial, breast, or ovarian cancer (Y. Wu et al., 2017; L. Yang et al., 2013).

1.3.4.1 **Bone protective effects of icariin**

The effect of icariin in osteoblastogenesis and osteoclastogenesis has been widely studied in both clinical and preclinical studies. A daily doses of 60 mg icariin, 15 mg daidzein, and 3 mg genistein were shown to be effective in preventing bone loss in late postmenopausal women in a 24-month randomized, double-blind and placebo-controlled trial (G. Zhang, Qin, & Shi, 2007). Similarly, icariin was shown to increase BMD, bone microarchitecture, and bone properties at distal femur, proximal tibia, and lumbar spine in six-month-old female OVX SD rats (Zhou et al., 2019), three-month-old female OVX SD rats (G. W. Li et al., 2014) upon treatment for 12 weeks. Moreover, icariin induced estrogenic differentiation, bone morphogenetic protein-2 (BMP-2) and Runx2, prevented apoptosis in human fetal osteoblastic hFOB 1.19 cells (Liang et al., 2012), mouse osteoblasts MC3T3-E1 cells (J. Zhao, Ohba, Shinkai, Chung, & Nagamune, 2008), as well as BMSCs from 8-month old female ICR mice (Hsieh, Sheu, Sun, Chen, & Liu, 2010). Furthermore, our group evinced that icariin modulated the estrogenic bone remodelling by upregulating the expression of estrogen-sensitive bone markers, OCN, ALP, and OPG, while downregulating the expression of RANKL in UMR-106 cells (Mok et al., 2010). Moreover, icariin impeded osteoclastogenesis by inhibiting bone resorption pit formation in osteoclast RAW264. 7 cells (Kim, Lee, & Park, 2018). Most importantly, no incidence of breast cancer, endometrium cancer (Zhou et al., 2019) and cardiovascular events was reported in the postmenopausal women after two years of icariin treatment, suggesting that icariin could exert tissue-specific effects in bone (G. Zhang et al., 2007).

1.3.4.2 **Activation of estrogen signaling by icariin**

Bone protective activities of icariin might be ER-dependent or ER-independent. Icariin could ER-independently activate different signaling pathways, including JNK/p38 (Xue et al., 2016), Wnt/β-catenin/BMP-2 (S. Fu, Yang, Hong, & Zhang, 2016), and more recently cAMP signaling (Shi et al., 2017) pathway in primary cilia of osteoblasts. On the other hand, recent studies reported that icariin exerted bone protection via the Wnt/β-catenin pathway in an ER-dependent manner in rat BMSCs (Q. Wei et al., 2016). *In vitro* studies also showed that MPP (selective ER-α antagonist), but not PHTPP (selective ER-β antagonist) could abolish the stimulatory effects of icariin on ALP activities in osteoblastic cells, suggesting that ER-α selectively mediated the estrogenic actions of icariin. Our recent study further showed that the rapid phosphorylation of ER-α(Ser118) and (Ser167) in osteoblastic cells induced by icariin was dependent on MAPK/ERK and phosphoinositide 3-kinases (PI3K)/Akt signaling pathways, respectively. Most importantly, the study showed that rapid membrane-initiated ER-α signaling pathways are involved in mediating the osteogenic and anti-apoptotic effects of icariin in osteoblastic cells (Ho, Poon, Wong, Qiu, & Wong, 2018). Taken together, these studies suggest the ER-dependence of icariin action in bone. Surprisingly, we found that icariin did not bind to ER-α and ER-β in the competitive binding assay (Ho et al., 2018), suggesting that the ER-dependent rapid estrogen signaling activation by icariin might be ligand-independent. These studies indicated that other unknown effectors might be involved to cooperate with ER-α66 in mediating the bone protection of icariin.

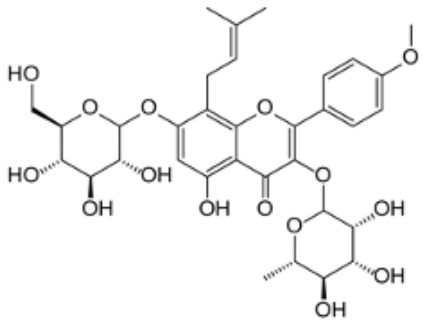


Figure 1.7 Structure of icariin. (Adapted from Qiu et al., 2008)

1.4 Estrogen receptors (ERs) and estrogen signaling

Estrogen receptor α (ER-α or now called ER-α66) and estrogen receptor β (ER-β) are the classical ERs responsible for E_2 action. ER-α66 was first identified in 1958, while ER-β was discovered in 1996. The genes for two ERs are located on separate chromosomes where ESR1 encodes ER-α66 or ESR2, respectively (Fuentes & Silveyra, 2019). The actions of estrogen are mediated through its binding to ERs on the cell surface, in the cytoplasm or on the nuclear membrane. Traditionally, estrogen could activate two types of estrogen signaling via these ERs, namely genomic estrogen signaling and rapid estrogen signaling (Fuentes & Silveyra, 2019; Yaşar, Ayaz, User, Güpür, & Muyan, 2016).

1.4.1 The general structure of ERs

ERs are class I nuclear receptors (Beato, 1989) that contains six domains including N-terminal domain (domain A/B), DBA binding domain (DBD, or conserved C domain), hinge region (domain D), ligand-binding domain (LBD. domain E) and domain F (Figure 1.8).

N-terminal domain is the less structured domain that could promote access for molecular recognition or ligand binding by expressing the capable surfaces for specific binding. Upon ligand binding, ERs undergoes a conformational change to employ general transcription machinery alone with or without other modulators (Celik, Lund, & Schiøtt, 2007). Transactional domain activation factor 1 (AF-1) is constructed in this domain and claimed as the centre of phosphorylation sites for primarily rapid estrogen responses. Fourteen phosphorylation sites in this region have been well investigated, including, Ser118 and Ser167 (Rastinejad, 2001).

DNA binding domain (Schwabe, Chapman, Finch, & Rhodes, 1993), contains two functionally distinct zinc fingers possessing a P box and D box sequences. P box is responsible for the estrogen response element (ERE) interactions while the D box is responsible for the ER dimerization. ERE composes of a palindromic hexanucleotide 5′ AGGTCAnnnTGACCT 3′ (Kumar et al., 2011; Wood, Likhite, Loven, & Nardulli, 2001). Hinge region is in charge of the nuclear localization signal and connection between DBD and the multifunctional carboxyl-terminal E/F in ERs (Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996).

LDB is highly conserved across species and specific for binding to estrogen. This globular LBD structure harbours a hormone-binding site, a homo- or heterodimerization interface, activation factor 2 (AF-2), and co-regulator (activator and repressor) interaction sites (Kong et al., 2005). The AF-2 locates across the E domain and F domain at the C-terminal of ERs.

The F domain has once reported having a specific modulatory function that affects the agonist and antagonist effectiveness and the transcriptional activity of the bound ERs in cells (J. Yang, Singleton, Shaughnessy, & Khan, 2008). For example, tamoxifen, a selective ER modulator, is an agonist of ERs in bone and uterus but an antagonist in nerve and breast (Shiau et al., 1998). This phenomenon could be accounted for by the different positioning of helix 12 and the F domain of ER-α66 in each cell type (de Lera, Bourguet, Altucci, & Gronemeyer, 2007).

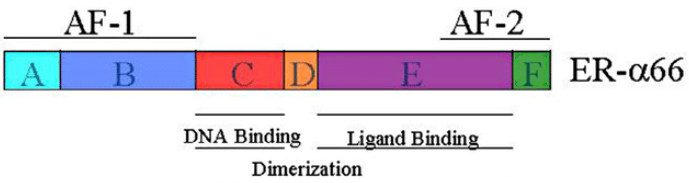


Figure 1.8 General structure of ER-α66 with six domains. (Adapted from Wang, 2015)

1.4.2 **Genomic estrogen signaling pathway**

Genomic estrogen signaling could be activated directly or indirectly (Figure 1.9). Direct genomic estrogen signaling is considered as the classical mechanism of estrogen signaling in which ERs form dimers upon binding to its ligands and translocate from the cytoplasm to the nucleus. E_2/ER complex acts as a transcriptional activator to bind ERE in the regulatory regions of estrogen-responsive genes followed by the recruitment of coregulators to the transcription start sites (Karin, Liu, & Zandi, 1997; Truss & Beato, 1993). Indirect genomic estrogen signaling are involved in regulation of estrogen responsive genes that lack the ERE sequences where ERs bind with transcription factors of the regulatory region via protein-protein interaction (Bajic et al., 2003). Transcription factors that involved in indirect genomic estrogen signaling include AF-1, stimulating protein-1 (Sp1), GATA binding protein 1 (GATA 1) (Burns & Korach, 2012; Yasar, Ayaz, User, Gupur, & Muyan, 2017).

1.4.3 **Rapid estrogen signaling pathway**

Rapid estrogen signaling pathways could be classified as ligand-dependent or ligand-independent. Ligand-dependent nongenomic signaling is initiated by binding of estrogen to membrane ERs which subsequently induces the mobilization of intracellular calcium, stimulation of adenylate cyclase activity, cAMP production as well as activation of MAPK and PI3K (Simoncini & Genazzani, 2003). On the other hand, ligand-independent signaling could be activated in the absence of estrogen in which the ERs are phosphorylated on specific residues which subsequently trigger secondary responses, including protein kinase A (PKA) or protein kinase C (PKC), cytokines. Peptide growth factors represent an especially vital group of estrogen-independent ER activators that include epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) (Nilsson et al., 2001; Vrtačnik, Ostanek, Mencej-Bedrač, & Marc, 2014).

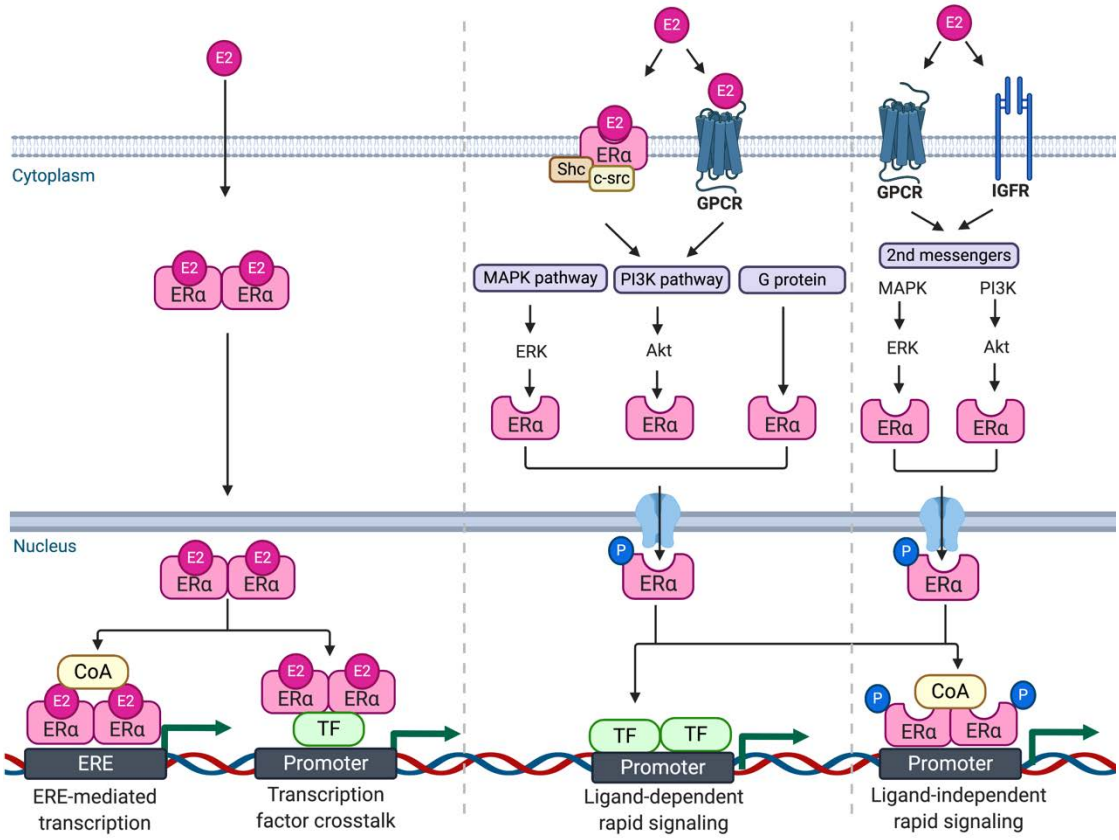


Figure 1.9 Genomic signalling and rapid signalling activated by estrogen.

There are two types of estrogen signaling, genomic and non-genomic signaling. Upon ligand binding, ERs undergo dimerization, translocate to the nucleus and bind to ERE to activate the transcription of the downstream gene (**Direct ligand-dependent genomic signaling**). ERs dimer can also recruit other transcriptional factors to promote or inhibit gene expression (**Indirect ligand-dependent genomic signaling**). In non-genomic signaling, ER could trigger rapid estrogenic signaling by phosphorylating secondary effectors that could lead to rapid changes in cells and genomic changes (**Ligand-dependent rapid signaling**). Without ligand binding, ERs can be activated by phosphorylation and mediate gene expression without binding to the ERE sequence (**Ligand-independent rapid signaling**).

1.5 Novel estrogen receptors

The classical nuclear ERs, ER-α66, and ER-β, have been regarded as sole ERs attributing to estrogen signaling (Fuentes & Silveyra, 2019). However, certain aspects of estrogen biology remain inconsistent with the action of these receptors. Previous studies indicated that ER-α66 knockout mice with neo cassette replacement in the AF-1 domain of ER-α66 retain some nongenomic estrogenic responses (Q. Gu, Korach, & Moss, 1999). A pure ERs antagonist, ICI 182,780, failed to block catecholestrogen-induced estrogen-responsive genes in the uterus of ER-α66 knockout mice (Das et al., 1997). Also, ICI 182,780 has been reported to promote the growth of estrogen-sensitive hippocampus neuron (L. Zhao, O'Neill, & Brinton, 2006) and human breast cancer cells (J. Wu, Liang, Nawaz, & Hyder, 2005; X. Zhang, Ding, Kang, & Wang, 2012). Rapid estrogen signaling, such as MAPK/ERK, could be activated in response to E_2 in native non-transfected, ER-α66-negative Chinese hamster ovary CHO-K1 cells, Rat2 fibroblasts cells, and monkey kidney fibroblasts COS-7 cells (Nethrapalli, Tinnikov, Krishnan, Lei, & Toran-Allerand, 2005). These findings indicated that unknown E_2 responders or ERs without AF-1 domain might exist and participate in estrogen signaling.

1.5.1 Estrogen receptor α 36 (ER-α36)

Estrogen receptor-α36 (ER-α36) is a new isoform of ER-α66, first described by Professor Wang Zhao-yi in 2005. It is synthesized by alternative splicing of ESR1 and transcribed from a previously unidentified half-ERE-containing promoter in the first intron of ER-α66 (Z. Wang et al., 2005). mRNA of ER-α36 is composed of exon 2-6 of the ESR1 gene with 27 amino acids at the C-terminus (Figure 1.10) (Zou, Ding, Coleman, & Wang, 2009). Structurally, the lack of ER-α66 conserved AF-1 containing domain A/B and AF-2-containing domain E/F in ER-α36 results in its loss of ability to mediate transcriptional activation, but retains the abilities for partial dimerization and DNA-binding. Different protein configuration of ER-α36 allows distinct ligand-binding spectrum and binding affinity when compared to ER-α66. The nuclear export signals within a unique 27 amino acid at the C-terminus drive ER-α36 to locate in the cytoplasm or cell membrane without any stimulation (Z. Wang et al., 2005).

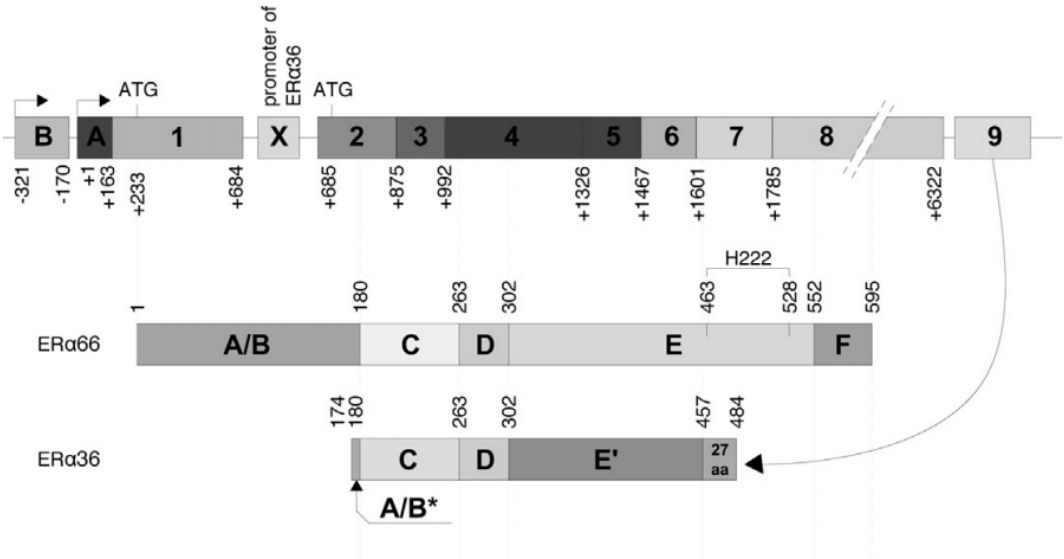


Figure 1.10 mRNA of estrogen receptor ER-α66 and ER-α36.

ER-α36 lacks AF-1 and NTD in the A/B domain and part of AF-2 and LBD in the E/F domain of ER-α66. In addition, 27 amino acids are specific to ER-α36. This specific sequence is encoded in exon 9 in the ERS1 gene. The expression of ER-α36 does not utilize the seven promoters upstream but starts from a promoter in the unknown intron 1 of ERS1. In addition, several transcriptional factors could bind to this unknown promoter, like, Sp1, AhR, WT-1, AP-1, Erg-1, NFkB, GATA-1. This promoter contains an imperfect ERE sequence which could be activated by other ERs. (Adapted from Sołtysik, 2015)

1.5.1.1 **The physiological role of ER-α36 in estrogen-sensitive tissues**

ER-α36 could be found in different estrogen-responsive tissues, including reproductive tissues (ovary, uterus, breast and testis) as well as non-reproductive tissues (endothelial and vascular smooth muscle cells, kidney, cartilage, bone, lung and heart). More specifically, it is found in the pyramidal neurons and the hippocampus and cortex of neonatal and adult SD rats, both ER-α66-positive cells (human endometrial cancer Ishikawa cells, breast cancer MCF-7, HB3396, and T47D cells, neuroblastoma SHSY-5Y cells) and ER-α66 negative cells (human triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells) (Soltysik & Czekaj, 2015).

Recently, ER-α36 is found to play a critical role in estrogen-related diseases. ER-α36 could mediate the development of estrogen-dependent cancers and tamoxifen-resistance of breast cancer (Lin et al., 2010). An increase in ER-α36 is associated with the occurrence of endometrial cancer (PENG et al., 2012), gastric carcinogenesis (Z. Fu et al., 2013), reduction of tamoxifen sensitivity to breast cancer, prevention of apoptosis of human neuroblastoma (H. B. Wang, Li, Ma, & Zhi, 2018).

ER-α36 might also be associated with bone health. Temporary expression of ER-α36 in human bone marrow-derived stromal cells seems to be colocalized with Runx2 for osteoblast differentiation (Francis et al., 2014). ER-α36 expressions in osteoblast, osteoclasts, and bone marrow tissue of normal premenopausal women are higher than that in Chinese osteoporotic postmenopausal women, suggesting that the decrease in ER-α36 might be associated with the occurrence of postmenopausal osteoporosis. The expression of ER-α36 positively correlates with BMD and negatively with bone turnover markers collagen type 1 cross-linked N-telopeptide (P1NP) and OCN in Chinese postmenopausal women. In the same study, knockdown of ER-α36, however, further increased the mRNA expression of E_2-induced bone formation markers (ALP and OCN) in osteoblasts isolated from postmenopausal women (Xie et al., 2011). Thus, ER-α36 appears to play a role in maintaining bone health, but the role of ER-α36 in bone remodelling in still far from clear.

1.5.1.2 **Estrogen signaling mediated by ER-α36**

E_2 is a natural ligand for ER-α36 with the reported dissociation constant (Kd) for E_2 is weaker than that of ER-α66 (Kd = 0.2–0.3 nM). Binding of E_2 to ER-α36 would trigger the downstream secondary messenger-dependent pathways (Lianguo Kang et al., 2010). Zhang et al demonstrated that rapid estrogen signaling could be activated in ER-α66 expression knocked-down cells but not in ER-α36 knockdown cells, indicating that ER-α36, without AF-1 and AF-2, could mediate rapid estrogen signaling (Z. Fu et al., 2013).

ER-α36 could either activate or deactivate the MAPK/ERK pathway as ERK2 could directly bind to the domain D of ER-α36, which in turn is recognized by its activators, substrate, and regulators for both phosphorylation and dephosphorylation (Omarjee et al., 2017). E_2 was reported to recruit ER-α36 to stimulate the phosphorylation of ERK and Akt pathway in triple-negative breast cells (X. T. Zhang et al., 2011) and osteoblast isolated from postmenopausal women within 15 minutes (Xie et al., 2011). Moreover, ER-α36 is reported to be involved in icaritin-induced growth inhibition of triple-negative breast cancer cells via activation of MAPK/ERK (X. Wang et al., 2017). Furthermore, it could trigger ERK phosphorylation in ER-α36-positive HEK293 cells (Z. Wang et al., 2006), ER-α66-negative breast SK-BR-3 cells (Pelekanou et al., 2012) as well as ER-α66 knockdown endometrial cancer Hec1A cells (Lin et al., 2010). The phosphorylation of ERK-mediated by ER-α36 upon treatment with E_2 could last for 6 hours in osteoclast from non-osteoporotic postmenopausal (Xie et al., 2011).

ER-α36 is reported to interact with the epidermal growth factor receptor (EGFR) at the basal state and gradually dissociate from EGFR and associate with Src and Shc upon binding to E_2. In addition, ER-α36 is involved in the rapid estrogen signaling in association with insulin-like growth factor 1 receptor (IGF-IR), EGFR, and HER2 in ER-positive breast cancer cell MCF-7 cells. ER-α36 is also shown to activate MAPK/ERK pathways via PKC-gamma in the endometrial cancer cell to promote cell proliferation (Omarjee et al., 2017; Tanoue, Adachi, Moriguchi, & Nishida, 2000).

The sensitivity of ER-α36 toward E_2 is found to be more potent than that of ER-α66. Phosphorylation of ERK by ER-α36 is triggered upon a broad concentration range of E_2 (10^{-16}–10^{-6} M) in breast cells, which covers the physiological levels of E_2 in women at different periods of life (Lianguo Kang et al., 2010). Moreover, study by Xie et al. revealed that ER-α36 mediated a rapid and transient activation of the MAPK/ERK pathway in osteoblasts, as well as a strong and prolonged activation of the MAPK/ERK pathway in osteoclast induced by low-level E_2 (10 pM), but its effect is diminished at higher concentration of E_2 (Lianguo Kang et al., 2010). This finding suggested that higher postmenopausal ER-α36 expression might substitute ER-α66 and account for the rapid estrogen signaling at low E_2 levels. Taken together, ER-α36 seems to bind to E_2 and mediate ligand-dependent rapid estrogen signaling, namely MAPK/ERK or EGFR/IGF-1R pathway. However, it is still unknown if ER-α36 cooperates with ER-α66 in mediating the bone protective effect of icariin and EXD at low-E_2 level.

1.5.2 **G-protein coupled estrogen receptor (GPER)**

G protein-coupled estrogen receptor 1 (GPER) (also known as G protein-coupled receptor 30, GPR-30) possess structural and functional characteristic shared by the members of the G-protein-coupled receptor (GPCR) superfamily, the largest class of plasma membrane receptors (Gaudet, Cheng, Christensen, & Filardo, 2015). It is proposed that the translocation of GPER from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane is necessary for signaling activation. The plasma membrane is primarily regarded as the subcellular site of action for GPCR. Upon engaging their cognate ligands, activated GPCRs trigger a variety of signaling cascades, and subsequently undergo receptor endocytosis to avoid excessive signaling, a process referred to as receptor desensitization (L. Wang, Martin, Brenneman, Luttrell, & Maudsley, 2009).

1.5.2.1 **The physiological role of GPER in estrogen-sensitive tissues**

GPER expression was found in many tissues, including endometrium, ovarian, breast, brain, skin, brain. The expression and signaling activation of GPER positively correlates with advanced disease and worsen prognosis (Gaudet et al., 2015). First, GPER was claimed to stimulate aromatase activity and positively associated with breast tumor size, Her2/Neu expression, and the presence of metastases (Hsu, Chu, Lin, & Kao, 2019). GPER deficient mice also expressed dysregulated metabolic functions like increase visceral adiposity and obesity (Meyer, Clegg, Prossnitz, & Barton, 2011). In the brain tissue, G-1, a GPER-specific agonist, could delay the loss of hippocampal neurons (Lebesgue et al., 2010). Knockout of GPER could abrogate E_2-induced protection of hippocampal CA1 neurons against global cerebral ischemia (Kosaka et al., 2012). G-1 was also reported to activate membrane-ERs and contribute to the estrogen-mediated modulation of dopamine transporters in PC12 pheochromocytoma cells (Alyea et al., 2008). GPER was suggested to promote estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting B-cell lymphoma 2 (Bcl-2) expression in keratinocytes (Kanda & Watanabe, 2003).

The role of GPER in bone remodelling is controversial. The bone-preserving raloxifene and tamoxifen are known to be GPER agonists which indirectly suggests the role of GPER in bone metabolism (Ohlsson & Vandenput, 2009). GPER knockout mice was reported to lack normal regulation of the epiphyseal growth plate and estrogen-mediated insulin-secretion, suggesting that GPER is required for the healthy development of the growth plate (Mårtensson et al., 2009; Windahl et al., 2009). However, GPER knockout in male mice shows a greater femur length, femoral area bone mineral, and trabecular bone volume than the wild type mice (Ford et al.,

2011; Windahl et al., 2009). In contrast, GPER-antagonist G1 administration did not influence tibia and femur growth in OVX C57BL/6 mice (ref). Taken together, these results suggest that GPER may involve in the regulation of bone mass, size and microarchitecture, as well as the remodelling of skeletal tissue. However, the exact role of GPER in bone remodelling requires further investigation.

1.5.2.2 **Estrogen signaling mediated by GPER**

GPER was found to mainly mediate nongenomic estrogen signaling. Upon ligand binding, signaling occurs via heterotrimeric G protein activation resulting in matrix-metalloproteinase activation via EGFR transactivation with subsequent MAPK and Akt activation (Michaelson, Ahearn, Bergo, Young, & Philips, 2002). Transcriptional activation has also been reported secondary to kinase activation. GPER was found to activate ERK1/2 signaling in the nervous system (Prossnitz & Barton, 2011), ER-α66-related pathway in the uterus, PI3K/Akt pathway in the breast, and activating cAMP and calcium mobilization in breast and neuron (Gaudet et al., 2015). Also, G-1 treatment, an agonist of GPER, enhances the activation of pro-survival kinase, Akt, and ERK1/2 while antisense knockdown of GPER block these signaling events in neurons (Roque & Baltazar, 2019). Taken together, GPER seems to act as a “collaborator” of rapid estrogen signaling and might be a suitable candidate for mediating the rapid estrogen signaling in bone cells.

1.5.3 **Interaction among ERs**

1.5.3.1 **ER-α36 seems to be an inhibitor of ER-α66**

Structurally, ER-α36 contains a protein dimerization domain, which allows the formation of heterodimers ER-α66/ER-α36. It is reported that the heterodimer could prevent the translocation of cytoplasmic ER-α66 to the nucleus, which in turn inhibits ER-α66-mediated genomic signaling in MCF-7 cells. Also, ER-α36 shares a similar DNA binding domain with ER-α66, which leads to competition with ER-α66 on DNA binding sites. These studies proposed that the increase of ER-α36 expression might inhibit the transcription action of ER-α66 (Lee et al., 2008). Besides, the regulation of ERs expression is a key for modulating estrogen signaling. The expression of ER-α66 and ER-α36 seems to be oppositely regulated. The promoter activity of ER-α36 was suppressed by knockin of ER-α66 in HEK293 cells, likely via interaction with the imperfect ERE half site located upstream of the ER-α36 promoter sequence (Zou et al., 2009). Also, the knocked-down of the WT1 transcription factor in MCF-7 cells caused a reduction of ER-α66 expression but an increase in ER-α36 expression (L. Kang, Wang, & Wang, 2011). It indicates that the expression of ER-α66 and ER-α36 might be oppositely regulated.

1.5.3.2 **GPER could be a collaborator of ER-α36**

ER-α36 and GPER physically interact in human monocytes (Pelekanou et al., 2016). Also, the expression of ER-α36 is positively regulated by GPER. Kang reported that the knockdown of GPER expression in ER-negative breast cancer SK-BR-3 cells downregulated ER-α36 expression. Upregulation of the ER-α36 promoter activity by GPER might be mediated by cAMP/PKA/CREB or Src/MAPK/AP-1 (Lianguo Kang et al., 2010). In addition, ER-α36 is likely a downstream target gene of GPER as the knockdown of GPER could reduce both GPER and ER-α36 in SHSY-5Y cells (Han et al., 2015). E_2 stimulated cell proliferation in seminoma-like Tcam-2 cells via ER-α36 is reported to be GPER-dependent (Wallacides et al., 2012). G1, previously considered as GPER-specific agonist, could was shown to recognize and activate ER-α36-mediated rapid estrogen signaling in GPER-negative HEK293 cell and SK-BR-3 cell (Lianguo Kang et al., 2010). In other words, previously reported activities of GPER in response to E_2 might be mediated by its ability to induce ER-α36 expression.

Chapter 2. Hypothesis and Objectives

2.1 Hypothesis

Er-xian decoction (EXD) and its abundant flavonoid icariin, have been prescribed for the management of postmenopausal osteoporosis without severe side effects on reproductive organs. Extensive studies reported that they could tissue-selectively exert estrogen-like bone protection, probably via rapid estrogen signaling (J.-Y. Li et al., 2017; Y. Wang et al., 2019; G. Zhang et al., 2007). However, due to the complexity and interaction of the active components in EXD, the mechanisms behind the estrogenic bone protection of EXD is far from clear. Moreover, icariin is known to be a phytoestrogen, but its estrogenic actions seem to be ligand-independent as it fails to bind to estrogen receptor α 66 (ER-α66) nor estrogen receptor β in competitive binding assay (Ho et al., 2018). It is crucial to study the mechanism by which EXD and icariin activate estrogen signaling in achieving their tissue-selective effects in bone, and investigate if other unknown effectors might be cooperating with ER-α66 in mediating their bone protection. Recent studies have discovered two novel membrane associated ERs, estrogen receptor α 36 (ER-α36) and G-protein coupled estrogen receptor (GPER) which mainly exert rapid estrogen signaling in response to estrogen and might play a role in bone remodelling (Gaudet et al., 2015; Z. Wang et al., 2005).

We hypothesized that ER-α36 and GPER might involve in mediating the rapid estrogenic effects of icariin and EXD in bone through crosstalk with ER-α66. It is hoped that this project could provide new insights to understand the mechanisms involved in mediating the effects of icariin and EXD on bone protection.

2.2 Objectives

This project aimed to study the involvement of novel estrogen receptors in mediating rapid estrogenic effects of Er-Xian Decoction and icariin in bone. The objectives are:

1. To characterize the tissue responsiveness to E_2 and icariin in bone: expressions of different estrogen receptors
2. To characterize the role of GPER and ER-α36 in the bone protective effect of icariin
3. To characterize the involvement of GPER and ER-α36 in the bone protective effect of EXD
4. To characterize the ER-α66 independence in the bone protective effect of icariin and EXD

Chapter 3. The tissue responsiveness to estrogen in bone upon treatment with E_2 and icariin: expressions of different estrogen receptors

3.1 Introduction

Postmenopausal osteoporosis is the most common type of osteoporosis. Estrogen deficiency induced by ovary depletion causes the increase in bone resorption without a corresponding increase of bone formation, leading to uncoupled bone remodelling and a net loss of bone in female (Dobbs et al., 1999; Watts, 2018). Hormone Replacement Therapy (HRT) (Marco Gambacciani & Levancini, 2014) and selective estrogen receptor modulators (SERMs) were being used as the clinical treatment for postmenopausal osteoporosis (Komm & Chines, 2012). However, these therapies are associated with severe side effects, including pulmonary embolism, venous thromboembolism, heart attacks, breast cancer, endometrial cancer, and colorectal cancer. Thus, alternative approaches for the management of menopausal symptoms and estrogen-related diseases in postmenopausal women are needed.

Icariin, a plant-derived non-steroid flavonoid glucoside, is considered as a phytoestrogen with the ability to mediate estrogen-related pathways (M. S. Wong & Zhang, 2013). Our previous studies suggested that icariin could promote osteogenic proliferation, alkaline phosphatase (ALP) activity and its mRNA expression in osteoblasts. The bone protection of icariin might be mediated by estrogen receptor alpha 66 (ER-α66)-dependent and estrogen receptor beta (ER-β)-independent rapid estrogenic signaling, like MAPK/ERK, PI3K/Akt in murine pre-osteoblastic MC3T3-E1 cells and rat osteoblast-like UMR106 cells without activation of estrogen responsive element (ERE)-mediated genomic event (Ho et al., 2018; Mok et al., 2010; Zhou et al., 2019). Interestingly, a competitive binding assay confirmed that icariin does not bind to ER-α66 (Ho et al., 2018). Therefore, the estrogenic bone protective effects of icariin are ligand-independent and ERE-independent and might involve other receptors that cooperate with ER-α66 to mediate the effect of icariin.

ER-α66 (also known as ER-α) and ER-β are not the sole estrogen receptors. Recent studies indicated that unknown proteins or ERs without AF-1 domain might involve in activating estrogenic signaling and respond to anti-estrogen (Z. Y. Wang & Yin, 2015). G-protein coupled estrogen receptor (GPER), and estrogen receptor alpha 36 (ER-α36) are novel estrogen receptors which might mediate rapid estrogenic signaling. Upon ligand binding, GPER could activate multiple effectors, including cAMP, Ca^{2+}, and c-Src, which is followed by the induction of metalloproteinases and subsequent activation of downstream signaling molecules, including MAPKs and PI3Ks (Gaudet et al., 2015; Michaelson et al., 2002). On the other hand, ER-α36 is a novel isoform of ER-α66 transcribed from a previously unidentified half-ERE-containing promoter in the first intron of ER-α66 (Z. Wang et al., 2005). Structurally, the lack of ER-α66 conserved domain A, B, F, and part of domain E in ER-α36 resulted in different

protein configurations, ligand binding spectrum and binding affinity compared to ER-α66 (Soltysik & Czekaj, 2015). Also, the nuclear export signals within a unique 27 amino acid at the C-terminus drive ER-α36 to locate in the cytoplasm and rest on the cell membrane without any stimulation (Wallacides et al., 2012; Z. Wang et al., 2005). Interaction of ER-α36 with other signal complexes (such as GPER, EGFR, Shc, Src) is needed to elicit cytoplasmic estrogenic pathways (Lianguo Kang et al., 2010; Omarjee et al., 2017). Collectively, both GPER and ER-α36 are considered as the novel types of extranuclear ERs mediating rapid estrogenic signaling (Z. Y. Wang & Yin, 2015).

The expressions of GPER and ER-α36 were also associated with bone growth. Knockdown of GPER in mice was discovered to increase bone mass, femur size, cortical thickness, and mineralization (Ford et al., 2011). Also, estrogen failed to reduce longitudinal skeletal growth in GPER knockout ovariectomized (OVX) mice (Windahl et al., 2009). The upregulation of ER-α36 was also found to be associated with the occurrence of postmenopausal osteoporosis in the clinical study (Xie et al., 2011). These studies suggest that extranuclear ER-α36 and GPER, beside classical ER-α66, could be suitable candidates for mediating rapid estrogenic signaling induced by icariin during osteoblastogenesis.

Crosstalk among ER-α66, ER-α36, and GPER have been revealed in mediating estrogenic actions. ER-α66 could suppress the expression of ER-α36 by targeting the half-ERE sequence in its promoter while ER-α36 could inhibit ER-α66-induced genomic signaling by retaining ER-α66 in the cytoplasm in form of ER-α66/ER-α36 heterodimer (L. Kang et al., 2011; Zou et al., 2009). GPER expression is found to be positively associated with that of ER-α36. Also, ER-α36 is considered as the downstream effector of GPER to elicit nongenomic estrogenic signaling in breast cells (Z. Fu et al., 2013; Wallacides et al., 2012). Thus, it is possible that the rapid estrogenic responses in bone might be resulted from the coordinated regulation of different ERs. However, the crosstalk of extranuclear ER expression in response to estrogen or icariin alone in bone are still unclear.

This chapter aimed to study the tissue responsiveness of osteoblast to estrogen via the regulation of ERs expression and the relationship amongst these ERs in response to icariin or estradiol (E_2) treatments. First, the expression of ERs in OVX rats and ER-α66-positive osteoblasts upon treatment with icariin or E_2 was studied. Second, the regulation of novel ERs expression by icariin and E_2 were also studied using ER-α66-negative human embryonic kidney 293 (HEK293) cells. These studies could allow us to determine the receptor crosstalk in mediating the action of icariin and E_2 in bone.

3.2 Methodology

3.2.1 *In vivo* study on rats

3.2.1.1 Experimental designs

The present experiment was conducted under the animal license issued by the Department of Health, Hong Kong Special Administrative Region Government, and the Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (animal license No. 16-129; ASESC Case: 13/18). Sixty 6-month-old female Sprague-Dawley rats were purchased from The Chinese University of Hong Kong and housed in centralized animal facilities of The Hong Kong Polytechnic University on a 12-hour light and dark cycle. Water and food were available ad libitum. After 1 week of acclimatization, all the rats were given bilateral OVX or sham operation. During the preliminary experiment, rats were allowed to consume diets ad libitum and the daily amount of intake was recorded for 5 days. Based on the preliminary study, the mean daily intake of diet for each rat was established as 15 g (the minimal amount of daily intake) in the present study. Upon recovery for 2 weeks, the OVX rats were randomly subjected to oral administration with vehicle (OVX), 17ß-estradiol (E2, 1.0 mg/kg/d), and icariin at 3 doses of 50 ppm (ICA-50 ppm, 0.05 g of icariin in 1 kg of diet, 2.5mg/kg/day), 500 (ICA-500 ppm, 0.5 g/kg diet in 1 kg of diet, 25mg/kg/day) and 3000 ppm (ICA-3000 ppm, 3.0 mg/kg diet in 1kg of diet, 150mg/kg/day) in the form of icariin-containing diets for consecutive 12 weeks. The sham-operated rats were administered vehicle (sham). During the whole recovery period and treatment, the rats in the sham, OVX, and E2 treatment groups were paired-fed the control phytoestrogen-free diet (AIN93-M) to remove the influences of phytoestrogens. There were 12 rats in each group. Upon sacrifice, left tibia heads were collected to study tissue responsiveness to estrogen by measuring ERs expressions in bone in OVX rats upon icariin treatment by western blotting.

3.2.1.2 Western blotting

Tibia heads from left legs were removed and individually immersed with 300µl lysis buffer (Beyotime, Shanghai, China, Cat# P0013) supplemented with protein inhibitors, 1mM phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Rockford, IL, United States, Cat#36978). in Precellys Tissue grinding CKMix50 lysis kit (Bertin Technologies, France, Cat#P000939-LYSK0-A) at 4ºC. Tibia heads in lysis kits were placed in Precellys Evolution and Cryolys (Bertin Technologies, France) and homogenized for 10 seconds with a 10-second interval for six times. Protein concentrations were determined using Bradford assay (Bio-Rad Laboratories, Inc., United States). 100µg protein lysates mixed with 5X reducing loading dye

and boiled for 10 minutes. Protein was loaded and separated by running 10% SDS-PAGE with 130V for approximately 2 hours. The gel was then transblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Danvers, MA, United States, Cat#IRVH00010) for 2 hours with 80V or overnight with 22V at 4°C. PVDF membrane was then blocked with 5% blotting-grade blocker (Bio-Rad Laboratories, Inc., United States, Cat#1706404) for 2 hours and subsequently probed overnight at 4°C with the following primary antibodies: rabbit anti-GPER (1:1000, Abcam, Cambridge, United Kingdom, Cat#ab39742), mouse anti-ER-α36 provided by Professor Wang Zhao-Yi from Beijing Shenogen Pharma Group Ltd. (1:1000), mouse anti-ER-α66 (1:1000, Santa Cruz, CA, United States, Cat#Sc-544) or mouse anti-β actin (1:2000, Thermo Fisher Scientific, Rockford, IL, United States, Cat#PA1-46296). The membranes were then washed three times with Tris-buffered saline with Tween 2(TBST) for 30 minutes in total. It is followed by incubation with anti-rabbit (1:1000, Santa Cruz, CA, United States, Cat# Sc-2004) or anti-mouse (1:1000, Cell Signaling Technology, Beverly, MA, United States, Cat#7076) IgG-HRP-conjugated secondary antibodies for 1 hour. After washing three times with TBST for 30 minutes in total, Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., United States, Cat#1705060) was added onto the membrane, and the chemiluminescence of the bound antibodies was visualized using Azure c600 (Azure Biosystems, United States). The intensities of the bands were quantified by ImageJ (LOCI, University of Wisconsin United States). mRNA expressions were normalized with control groups.

3.2.2 *In vitro* study

3.2.2.1 Experimental design

Mouse pre-osteoblast MC3T3-E1 cells, human osteoblast-like MG-63 cells, and ER-α66-negative human embryonic kidney 293 (HEK293) cells were employed. First, the basal mRNA expressions of ERs were measured in MC3T3-E1 cells and MG-63 cells as preliminary studies. Additionally, the interactions between these ERs were investigated in ER-α66-negative HEK293 cells. In this study, the basal expressions of other ERs were examined by western blotting. Overexpression of ER-α36, knockin of ER-α66 or GPER were performed in ER α66-negative HEK293 cells for studying the regulation of these ERs" expressions upon icariin treatment and binding affinity of icariin toward ER-α36.

3.2.2.2 Cell culture

MC3T3-E1 cells were cultured in alpha Modified Eagle Medium (αMEM, Life Technologies, Rockville, MD, United States, Cat# 41061) with 1% penicillin and streptomycin (Life Technologies, Rockville, MD, United States, Cat# 15140-122) supplemented with 10% fetal

bovine serum (FBS) (HyClone, Logan, Utah, United States, Cat# SV30160.03) in a humidified environment at 5 % carbon dioxide (CO_2) and 37 °C. After reaching 80% confluence, cells were passed once a week. For the experiments, cells were seeded at a quantity of approximately $15x10^4$ cells/well, 2.5×10^4 cells/well, and 1×10^4 cells/well into 6-well, 24-well and 96-well flat-bottomed cell culture plates, respectively. Besides, MG-63 cells and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Rockville, MD, United States, Cat#12800-017) supplemented with 1% penicillin and streptomycin supplemented with 10%FBS in a humidified environment at 5 % CO_2 and 37 °C. Cells were passed once a week. For the experiments, cells were seeded at a quantity of approximately 12×10^4 cells/well and 2×10^4 cells/well into 6-well and 24-well flat-bottomed cell culture plates, respectively.

3.2.2.3 **Transformation and transfection**

The expression vector of ER-α36 (CMV-36) and GPER (CMV-GPER) and control vector (CMV-HA) was kindly given by Prof. Wang Zhao-yi from Beijing Shenogen Pharma Group Ltd. (Figure 3.1). DH5α competent cells (Thermo Fisher Scientific, Rockford, IL, United States, Cat#18265017) were transformed with these expression vectors by heat shock. 5 µl of expression vectors were mixed with 50 µl of competent cells in a microcentrifuge or falcon tube. After incubation of the competent cell/DNA mixture on ice for 20-30 minutes, tubes were placed into a 42°C water bath for 45 seconds to perform transformation. Reactions were stopped by putting the tubes back on the ice for 2 min. After heat shock, 1ml Luria-Bertani broth (LB, Hopebio, Qingdao, China, Cat#HB0128) was added to the reaction tubes which were then shaken at 37°C for 45 minutes. To culture the recombinant cells, 50 µl reaction solution was placed onto a 10cm LB agar (Hopebio, Qingdao, China, Cat# HB0129) plate containing 100 µg /ml ampicillin and incubated at 37°C overnight. A single colony was picked up using sterile wooden sticks and transferred to 250µl LB solutions with 100 µg /ml ampicillin (Sigma-Aldrich, St. Louis, MO, United States, Cat# A9518). After shaking for 60 minutes at 37°C, 15ml LB broth was added to the reaction tube and incubated overnight at 37°C. The reaction was stopped on ice for 10 minutes. Expended recombinant cells were collected by centrifugation at 4000 rpm for 15 minutes. Plasmids from recombinant cells were extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Germantown, Germany, Cat# 27104).

MG-63 cells, MC3T3-E1 cells, and HEK293 cells were seeded in a 6-well plate for 24 hours with standard culture medium before replacing with phenol-red free (PRF) DMEM or αMEM without supplementation of FBS and antibiotics. After 24 hours, the cells were transfected with

1 μg CMV-36, 1 μg CMV-GPER, 0.4μg ER-α plasmid or 1 μg CMV-HA, by 4μl/well Lipofectamine ™ 2000 reagent (Thermo Fisher Scientific, Rockford, IL, United States, Cat#11668-019) in PRF medium without antibiotics and FBS for 24 hours. After 24 hours, the medium was removed and replaced with PRF-medium with antibiotics and FBS and cultured for 24 hours before cell treatment. The transfection efficiencies were examined by Western blotting and real-time PCR.

3.2.2.4 **Drug preparation**

17ß-estradiol (Sigma-Aldrich, St. Louis, MO, United States, Cat# E8875) was dissolved in absolute ethanol. Icariin (Biomol, Hamburg, Germany, Cat# I0901) was all dissolved in dimethyl sulfoxide (DMSO). After dissolved in their solvent, respectively, the drugs were sterilized by using a 0.22μm filter, followed by dilution with absolute ethanol. Concentrations of each drug used in the present study were determined based on the reference of previous studies.

3.2.2.5 **Real-time PCR**

RNA was extracted from treated cells with Trizol (Thermo Fisher Scientific, Rockford, IL, United States, Cat#15596018) by following the manufacturer's instructions. 2μg of total RNA was used to generate cDNA in a 20μl of RT reaction system by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific, Rockford, IL, United States, Cat#4368814) following the manufacturer's instruction. Real-time PCR was conducted in 96-well plate (The Applied Biosystems, Thermo Fisher Scientific, Rockford, IL, United States, Cat#N8010560) by Power SYBR™ Green PCR Mix (Thermo Fisher Scientific, Rockford, IL, United States, Cat#4367659) in PCR machines (QuantStudioTM 7 Flex Real-Time PCR System, The Applied Biosystems, Thermo Fisher Scientific, Rockford, IL, United States,) with specific primers listed in Table 4.1.

3.2.2.6 **ER-α36 promoter activity**

ER-α36 promoter activities were studied in ER-α66-negative HEK293 cells with knockin of ER-α66 or GPER. pER-α36-luc (kindly provided by Prof. Wang Zhao-yi) is a recombinant plasmid which contains the ER-α36 promoter sequence and a reporter gene which encodes firefly luciferase. HEK293 cells were seeded and cultured in a 6-well plate for 24 hours before replacing the growth medium with phenol-red free DMEM supplemented without FBS and antibiotics. After 24 hours, the cells were co-transfected with 0.4μg pER-α36-luc, 0.01μg pRL-TK (an internal control Renilla Luciferase vector for normalization) and 1 μg CMV-36, 1 μg CMV-HA, 1 μg CMV-GPER, or 0.4μg ER-α plasmid. The transfection into the cells was done

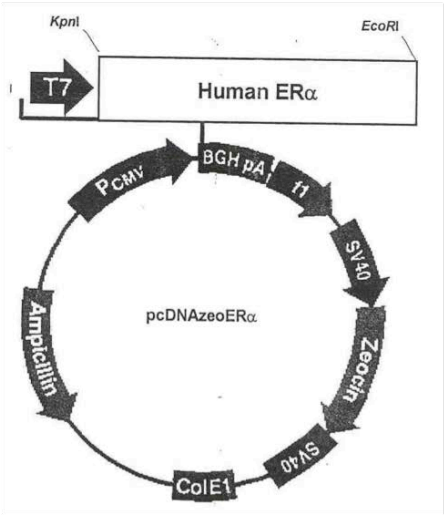
by combining the plasmids with Lipofectamine TM 2000 reagent (Invitrogen) and incubated in phenol red-free DMEM without any supplement for 6 hours. A vehicle, estradiol (10^{-8}M), icariin, and EXD at optimal doses were then added, followed by 24-hour incubation at 37°C. The cells were then lysed using Passive Lysis Buffer, and Dual-Luciferase Reporter Assay was carried out to study the dual-luciferase activities following the protocol (Promega, Ann Arbor, MI, United States, #TM040). The luminescent signals were detected using the Glomax-20/20 Luminometer (Promega, Ann Arbor, MI, United States). The ratio of the first reading (firefly luciferase activity) and second reading (Renilla luciferase activity) reflected the activities of the receptors in activating the ER-α36 transcription level.

3.2.2.7 **Statistical analysis**

Inter-group difference in both *in vitro* and *in vivo* study were determined by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. All graphs in this study were plotted by using GraphPad Prism Version 7.0.

(A) **ER-a66 expression provided by Dr. Vincent Giguere**

Plasmid: pCB6+ (Mammalian Expression)



(B) **CMV-ER-a36 expression provided by Prof. Wang Zhaoyi**

Plasmid: pCB6+ (Mammalian Expression)

ATGTACCCATACGATGTTCCAGATTACGC
T……GAATTCGCAAGGGAAGTATGGC
TATGGCTATGGAATC…..GTTTCCTCGTG
TGTCTAAAGCCTCTGGATCTC

HA tag :green
ER-α36 sequence :red
Ecoli restriction site: blue

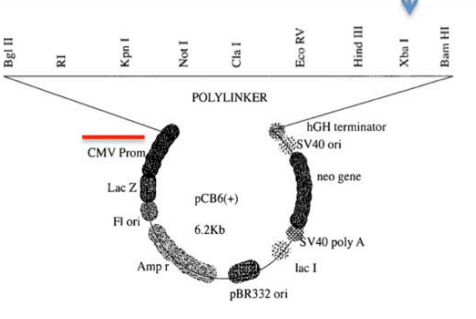


Figure 3.1The vector maps of (A) ERα plasmid (B) CMV-36 plasmid.

Table 3.1 Primer sequences for genes expressed in MG-63 cells and MC3T3-E1 cells

	MG-63 cells	MC3T3-E1 cells
Gene	Sequence	Sequence
ER-α66	GTCGGCGGGACATGCG TGCTTTGGTGTGGAGGGTCA	AGTGTCTGTGATCTTGTCCAG TGTGTGCCTCAAATCCATCA
GAPDH	AGGGCTGCTTTTAACTCTGGT CCCCACTTGATTTTGGAGGGA	TCAGGAGAGTGTTTCCTCGTC GGCCTCACCCCATTTGATGT
GPER	CTCTTCCCCATCGGCTTTGT TACAGGTCGGGGATGGTCAT	AACCTCACTGGGGACCTCTC CTCGCACGATGAGGGAGTAG

3.3 Result

3.3.1 Effect of icariin on different ERs protein expression in tibia heads of OVX rats

Fourteen weeks after ovariectomy, protein expressions of ER-α66, ER-α36, and GPER were significantly (P<0.001 vs. sham) increased in tibia head in adult female rats (Figure 3.2). Upon treatment with E_2 (1mg/kg.day) or icariin at 50, 500, and 3000ppm for twelve weeks, the protein expressions of ER-α66 in tibia head were significantly (P<0.001 vs. OVX) reduced in OVX rats. Only E_2 and icariin at 500ppm could remarkably (P<0.01 vs. OVX) reverse the OVX-induced increase in ER-α36 protein expressions in the tibia head of rats. Like E_2, icariin at 3000ppm significantly (P<0.05 vs. OVX) decreased GPER protein expression in tibia head in OVX rats.

3.3.2 Effect of icariin on ERs protein expressions in bone cells

As shown in figure 3.3, icariin mimicked the effect of E_2 to increase the protein expression of ER-α66 at 10^{-11}M, 10^{-9}M, 10^{-8}M and 10^{-7}M (P<0.05 vs. Control) and decreased the protein expression of ER-α36 and GPER at the dosages from 10^{-8}M to 10^{-6}M (P<0.05 vs. Control) in MC3T3-E1 cells. As for MG-63 cells, E_2 treatment dramatically elevated the protein expression of ER-α66 by1.5-fold (P<0.5 vs. Control), while icariin increased ER-α66 expression from 10^{-11}M to 10^{-8}M (P<0.5 vs. Control) upon treatment for 24 hours. In contrast, E_2 treatment remarkably (P<0.05) suppressed the protein expression of both ER-α36 and GPER by 0.8-fold in MG-63 cells upon treatment for 24 hours, while icariin could dose-dependently reduce the protein expressions of ER-α36 and GPER by around 0.7-fold (P<0.05 vs. Control) from the doses of 10^{-11}M to 10^{-8}M and reach the lowest at 10^{-11}M.

3.3.3 Effect of icariin on ERs mRNA expression in bone cells

Our results demonstrated that E_2 at 10^{-8}M and icariin at 10^{-8}M to 10^{-6}M significantly increased mRNA expression of ER-α66 in MC3T3-E1 cells upon treatment for 24 hours (P<0.05 vs. Control) (Figure 3.4). In addition, E_2 at 10^{-8}M and icariin at 10^{-6}M significantly boosted the mRNA expression of GPER in MC3T3-E1 cells. On the other hand, E_2 at 10^{-8}M and icariin at 10^{-11}M to 10^{-9}M significantly increased the mRNA expression of ER-α66, while both E_2 at 10^{-8}M and icariin at 10^{-11}M remarkably suppressed ER-α36 and GPER mRNA expression in MG-63 cells (P<0.5 vs. Control).

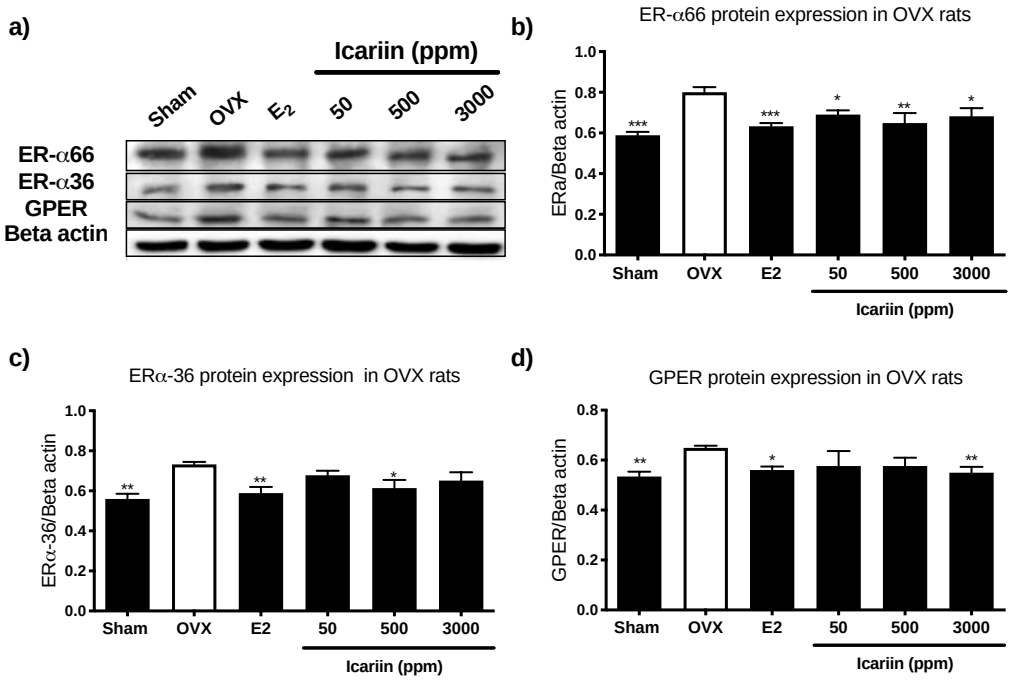


Figure 3.2 Effect of E_2 and icariin on ERs protein expressions in OVX rats.

Two weeks after ovariectomy, rats were orally administered with a vehicle, E_2 (1mg/kg.day) or icariin at 50ppm, 500ppm, and 3000ppm for 12 weeks. Tibia heads were isolated from rats upon sacrifice, and proteins were extracted to study the expression of ER-α66 (b), ER-α36 (c), and GPER (d) by western blotting. The figures are representative of three times of independent trials. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analysed by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. OVX.

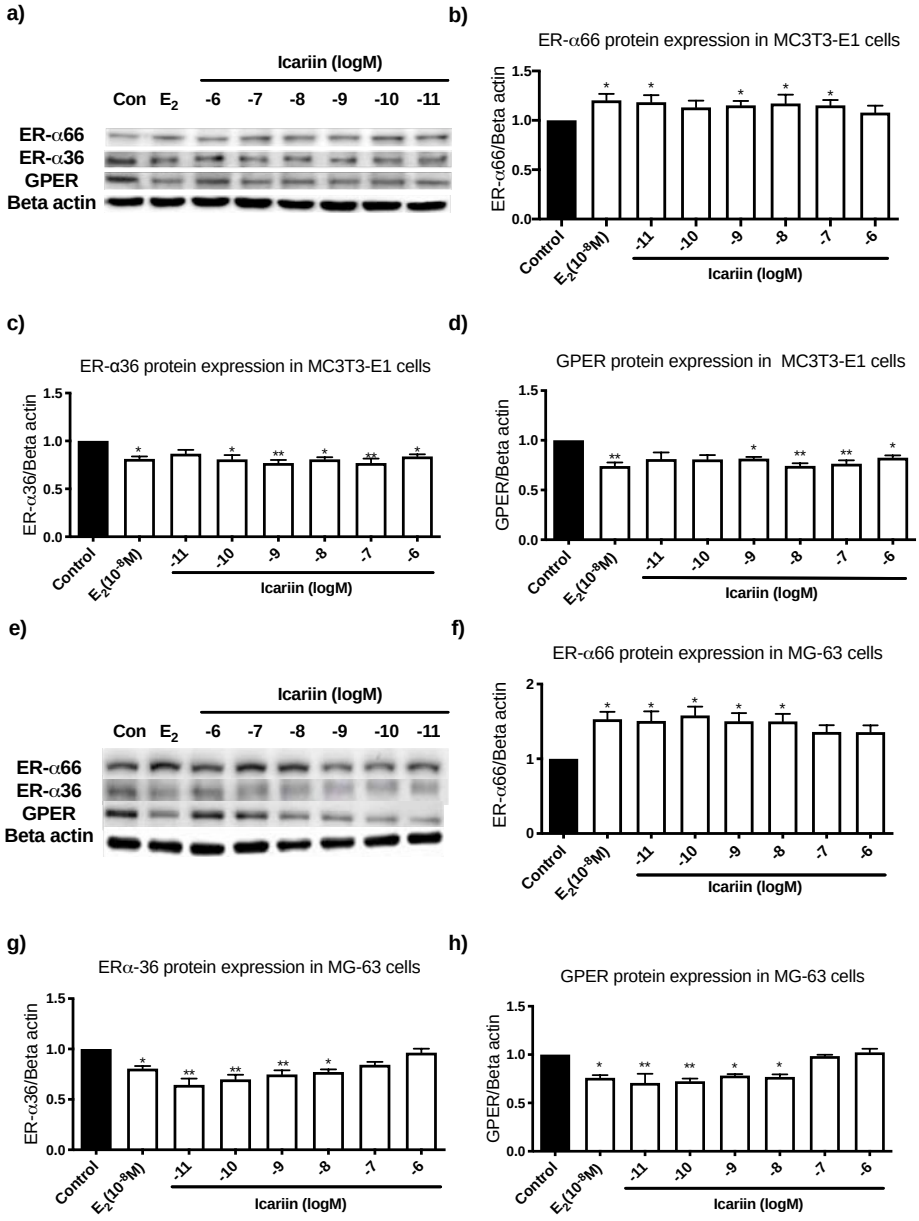


Figure 3.3 Effect of E_2 and icariin on ERs protein expressions in osteoblasts.

MC3T3-E1 and MG-63 cells were treated with vehicle, E_2 (10^{-8}M), or icariin (10^{-11}M-10^{-6}M) for 24 hours in phenol red-free DMEM containing 1% cs-FBS. Proteins were extracted to study the expression of ER-α66, ER-α36, and GPER in MC3T3-E1 cells (b-d) and MG-63 cells (f-h). The figures (a and e) are the representatives of immunoblot from three independent trials in MC3T3-E1 cells and MG-63 cells, respectively. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$ and ** $P < 0.01$ vs. Control.

3.3.4 Effect of GPER or ER-α66 knock-in and ER-α36 overexpression on ERs expression in human embryonic kidney (HEK) 293 cells

To evaluate the interactions amongst ERs, ER-α66 negative HEK293 cells were transfected with 1μg CMV-36, 1μg ER-α66 expression vector (ER-α66-V),1μg GPER-V or co-transfected with CMV-36 and ER-α66-V before 24-hour treatment with icariin (10^{-6}M) or E_2 (10^{-8}M). Our results showed that overexpression of GPER successfully increased not only GPER but also ER-α36 protein expressions in HEK293 cells (P<0.05 vs. Control). The increase of GPER and ER-α36 protein expressions induced by GPER overexpression were abolished by treatment with E_2 or icariin for 24 hours in HEK293 cells (P<0.05 vs. Treatment group transfected with control vector) (Figure 3.5). In addition, overexpression of ER-α66 in HEK293 cells significantly increased the expression of ER-α66 only (P<0.001 vs. Control), without altering the expression of ER-α36 or GPER protein. Such an increase in ER-α66 expression by overexpression was not altered by treatment with E_2 and icariin for 24 hours. On the other hand, overexpression of ER-α36 significantly induced the expression of ER-α36 protein, without altering the expression of ER-α66 or GPER protein in HEK293 cells (P<0.05 vs. Control). Such induction of ER-α36 protein expression was reversed by treatment with icariin and E_2 for 24 hours (P<0.05 vs. Treatment group transfected with the control vector). Moreover, dual overexpression of ER-α36 and ER-α66 could increase the expression of both receptors in HEK293 cells (P<0.01 vs. Control). Such an increase could be reversed by treatment with E_2, but not icariin, in HEK293 cells for 24 hours.

3.3.5 ER-α36 promoter activity in response to E_2 and icariin treatment in HEK293 cells with GPER or ER-α66 knock-in

In figure 3.6, overexpression of ER-α36 or in combination with ER-α66 enhanced basal ER-α36 promoter activities in HEK293 cell (P<0.001 vs. Control) while overexpression of ER-α66 slightly (P<0.05 vs. Control) suppressed it. Treatment with E_2 and icariin for 24 hours significantly reversed ER-α36 promoter activity induced by ER-α36 overexpression alone and co-transfection of ER-α36 and ER-α66 vectors, but not ER-α66 expression alone, in HEK293 cells (P<0.05 vs. Treatment group transfected with control vector).

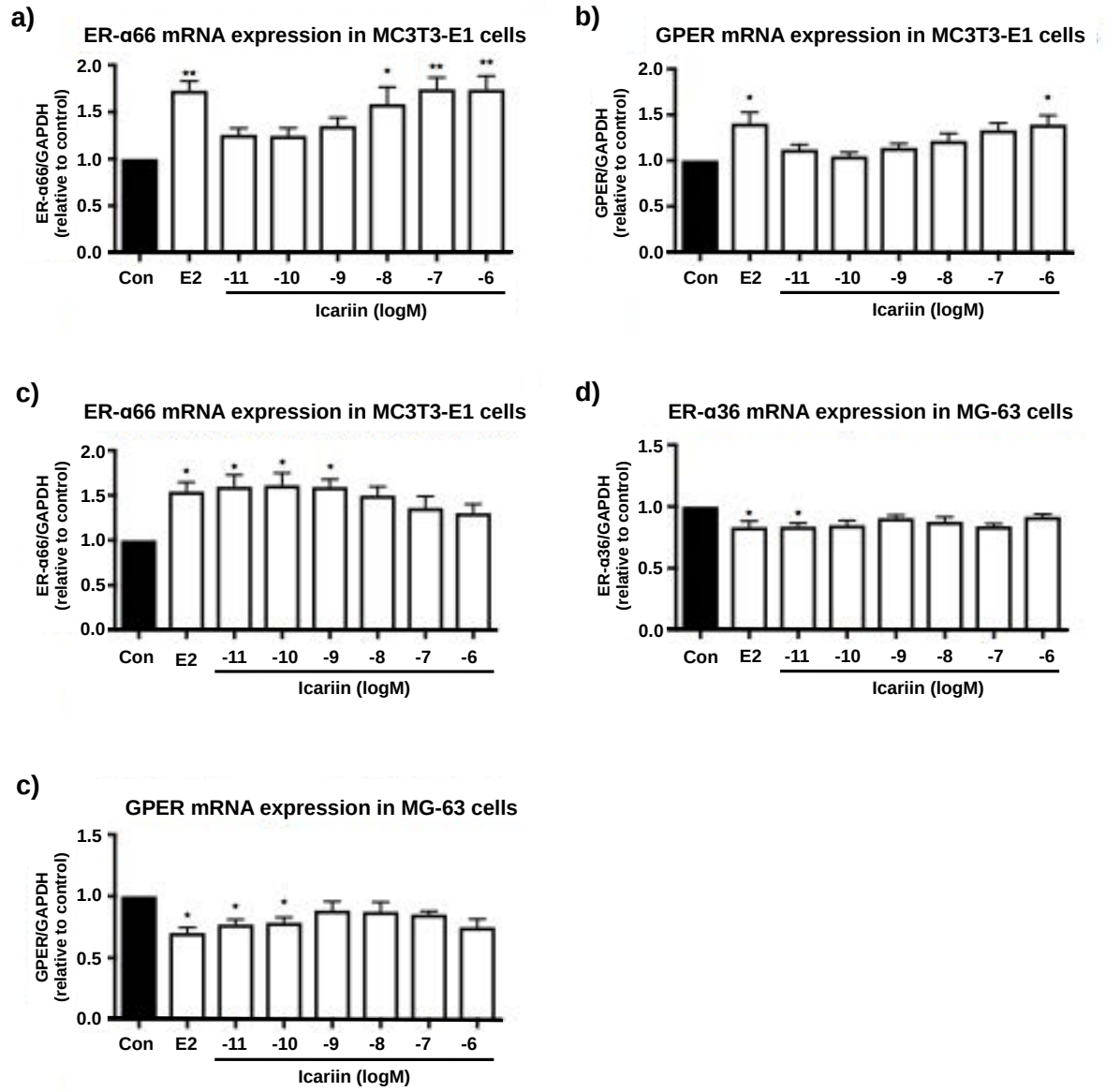


Figure 3.4 Effect of E_2 and icariin on ERs mRNA expressions in osteoblasts.

MC3T3-E1 cells and MG-63 cells were treated with vehicle, E_2 (10^{-8}M), or icariin (10^{-11}M-10^{-6}M) for 24 hours in phenol red-free DMEM containing 1% cs-FBS. mRNA was extracted by using Trizol reagent. mRNA expression of ER-α66 and GPER in MC3T3-E1 cells (a-b) and that of ER-α66, ER-α36, and GPER in MG-63 cells (c-e) were measured by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control.

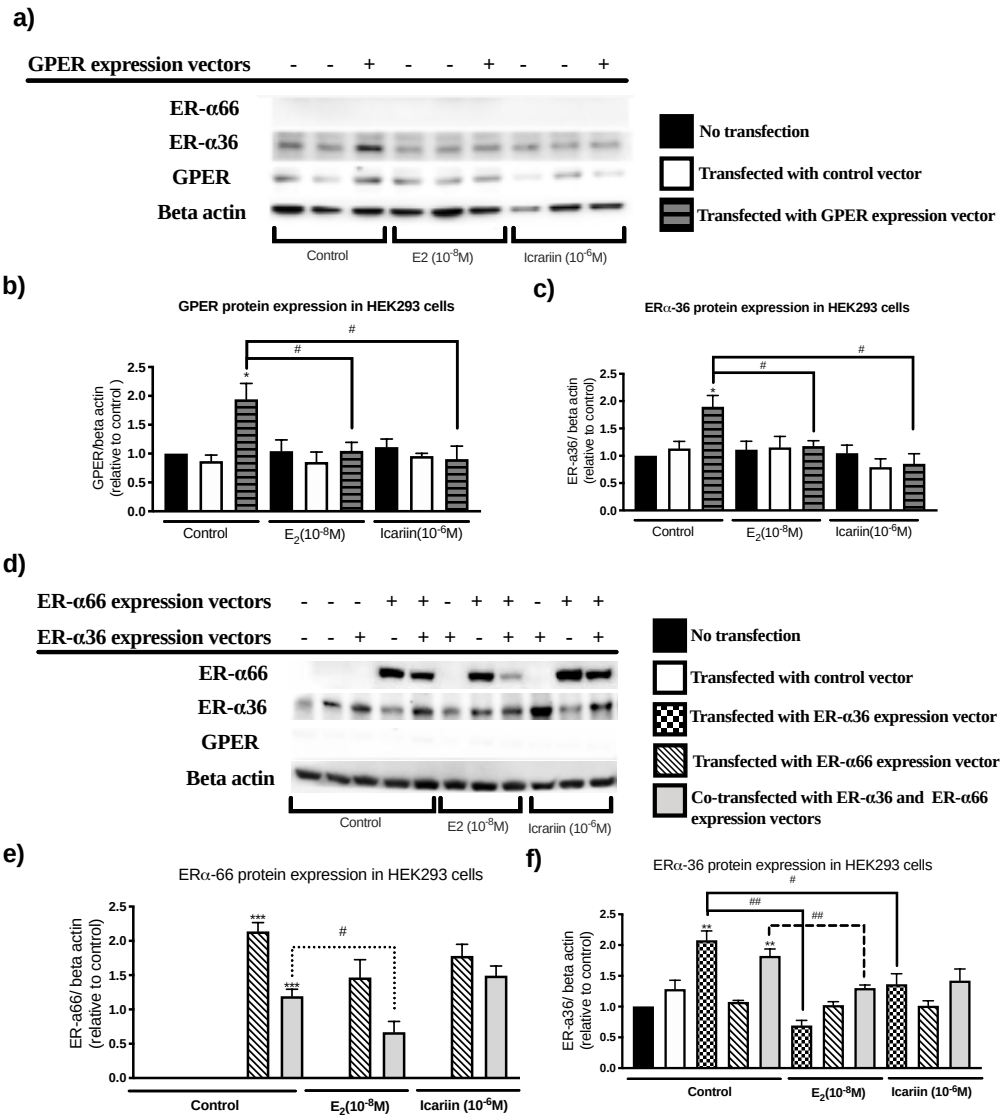


Figure 3.5 ERs expression in response to treatment with E_2 and icariin in HEK293 cells. HEK293 cells were transfected with 1mg CMV-36, 1mg ER-α66 expression vector (ER-α66-V), 1mg GPER-V, or co-transfected with CMV-36 and ER-α66-V before the 24-hour vehicle, E_2 or icariin treatment. Proteins were extracted, and the expression of ER-α66, ER-α36 and GPER in GPER-overexpressed (a-c) cells and ER-α66 alone, ER-α36 alone or dual overexpressed (d-f) in HEK293 cells were studied by western blotting. The figures are the representatives of three independent experiments in HEK293 cells. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Treatment group transfected with control vector.

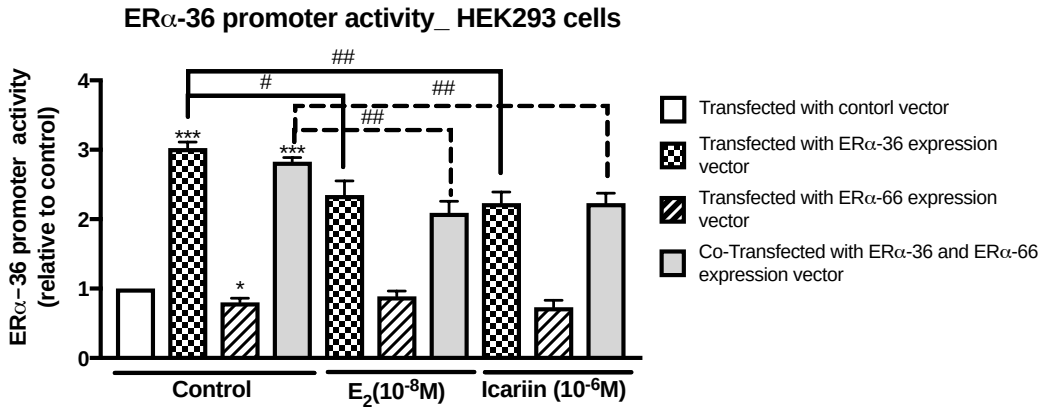


Figure 3.6 ER-α36 promoter activities in response to E_2 and icariin in HEK293 cells. HEK293 cells were co-transfected with 1mg pER-α36-luc, 0.04mg p-TK, 5mg CMV-36, 1mg ER-α66 expression vector (ER-α66-V) or combination of CMV-36 and ER-α66-V before the 24-hour vehicle, E_2 or icariin treatment. Dual-Luciferase Reporter Assay was used to monitor the activities of Firefly and Renilla luciferase induced by ER-α36 promoter. Data were presented by mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * P <0.05, ***P < 0.001 vs. Control; $^{\#}$ P <0.05, $^{\#\#}$ P <0.01 vs. Treatment group transfected with control vector.

3.4 Discussion

Here, we have attempted to understand the cooperative regulation of ERs in bone in response to E_2 or icariin. We studied the co-expression of ER-α36, ER-α66, and GPER in both *in vivo* and *in vitro*. It is found that the ER-α36 expression is differentially regulated by E_2 or icariin compared to ER-α66 but simultaneously modulated with GPER. Such regulation of ER-α36 expression by E_2 or icariin is GPER-dependent and ER-α66-independent.

ER-α66 is not the sole ER-α in bone

In this chapter, we elucidated that ER-α36 and ER-α66 co-exist in bone tissues (tibia head) of SD rats and osteoblastic cells (murine pre-osteoblast MC3T3-E1 cells and human osteoblast-like cells MG-63 cells). Ovariectomy could increase the expression of both ER-α36 and ER-α66 in tibia head. Expression of ER-α36 was reported in many estrogen-sensitive cells, including ER-positive breast cancer cells, MCF-7 , HB3396 , and T47D cells and ER-negative breast cancer cells MDA-MB-231 and MDA-MB-436 cells (Lee et al., 2008; Z. Wang et al., 2006), pyramidal neurons in hippocampal CA1 and CA3 (Kosaka et al., 2012), human neuroblastoma SHSY-5Y cells (H. B. Wang et al., 2018) and in primary osteoblast and osteoclasts from normal postmenopausal women (Xie et al., 2011). These evidences suggest that ER-α66 is not the sole ER-α in estrogen-sensitive cells.

Estrogen regulates ER-α36 expression

We next studied if E_2 could regulate ER-α36 expression in bone. Our results clearly showed that E_2 deficiency caused by OVX could significantly increase the protein expression of both ER-α66 and ER-α36, while E_2 treatment could reverse the effects of OVX on the expression of ERs in rats. It has been reported that the decrease in the expression of ERs might resulted from the loss of estrogen responsiveness in multiple tissues in rats. Therefore, the results from our *in vivo* study may suggest a feedback mechanism to promote the tissue sensitivity to the low level of E_2 production induced by OVX via increasing ERs expression in bone tissues. Moreover, our results indicated that E_2 could reduce both mRNA and protein expression of ER-α36 in MC3T3-E1 cells and MG-63 cells. Although the ER-α36 protein expression in response to E_2 *in vivo* was different from that in *vitro* study, ER-α36 was stated to be responsive to the E_2 level. This discrepancy of ER-α36 expression might due to complex cell content in the epiphysis (Bartelt et al., 2017; Hallett, Ono, & Ono, 2019) where bone marrow, osteoclast, osteoblasts, adipocytes and chondrocytes could be found. Therefore, It is more precise to study the effect of icariin and E_2 on bone formation in osteoblast.

Lower dose of icariin showed better effect on regulating ER-α36 expression than the higher dose *in vivo*

The estrogenic effect of icariin in bone was mediated by ERs, thus, the regulation of ERs expression by icariin might be closely related to the estrogenic effect of icariin in bone. In the present animal study, 500ppm icariin showed to have more potent effect than 3000ppm in terms of promoting bone mineral density, suppressing urinary DPD (bone resorption markers, unpublished) and modulating the expression of signaling molecules in various estrogen signaling molecules in bone marrow stromal cells analyzed by RNA sequencing (unpublished). Mostly importantly, higher concentration of icariin and its functional metabolites (icariside I and icariside II) were found in rat serum treated with 500ppm icariin when compared to 3000ppm. It implied that 500ppm icariin is more optimal for absorption and metabolising when compared to 3000ppm. Icariside I and icariside II were reported to have more potent bone protection when compared to icariin (Xiao et al., 2014) and might account for ERs expression. At gene level, our RNA sequencing results showed that 500ppm had more significant regulation of miRNA which are responsible for post-transcription modification of ERs. Let-7 miRNA family, which was found to suppress ER-α36 expression in human (Zhao et al., 2011), have been promoted by 500ppm icariin with 2.2 fold change and suppressed by 3000ppm with 0.00402 fold change (unpublished). Taken together, the potent effect of icariin at 500ppm on bone protection and micro RNA regulation and more diverse and rich metabolite found in serum might explain why 500ppm showed better effect on altering ER-α36 expression when compared to icariin at 3000ppm.

ER-α 36 and ER-α66 expression are differentially regulated

A bone-stage dependent doses of icariin were observed that lower doses of icariin seemed to be effective in MG-63 cells while higher doses of icariin was found to be effective in MC3T3-E1 cells. In particular , E_2 and icariin treatment could stimulate the expression of ER-α66 while suppressing ER-α36 mRNA and protein expression in both MC3T3-E1 cells and MG-63 cells. The differential regulations of these ERs are also reported by others in which the addition of E_2 could reduce ER-α36 expression while increased ER-α66 expression in osteoblasts harvested from postmenopausal women (Xie et al., 2011). ICI 182,780, an estrogen receptor-specific antagonist, could suppress the protein expression of ER-α66 while promoting ER-α36 expression in breast tissues (L. Kang & Z. Y. Wang, 2010). Moreover, Wilms Tümör 1, a dual transcription regulator, was reported to activate the promoter activity of ER-α66 while suppressing ER-α36 promoter activity in HEK293 cells (Zou et al., 2009).

Apart from their differential expression, their activations of subsequent signaling pathways upon stimuli were also different. ICI 182,780 was reported to block ER-α66-mediated classical and rapid estrogenic signaling in positive MCF-7 cells (Omarjee et al., 2017) and UMR 106 cells (K. C. Wong et al., 2014) in response to estrogen. However, ICI 182,780 could activate ER-α36-mediated ERK signaling in ER-α66-negative MDA-MD-231 cells (X. Zhang et al., 2012). This insensitivity of ER-α36 to ER-α66 blocker might due to the lack of helix 9-12 of ER-α66 in ER-α36 for ICI 182,780 binding (X. Zhang et al., 2012). Also, knockdown of ER-α66 in endometrium HEC-1-A cells could promote rapid estrogen signaling mediated by ER-α36 (Lin et al., 2009). In liver, E_2 was reported to promote apoptosis of hepatocellular carcinoma via ER-α66 while promoting its growth via ER-α36 in human hepatoma Hep3B cells (J. Chen et al., 2019). Based on these findings, it is likely that the expression of ER-α36 and ER-α66 are oppositely regulated and might be closely related to their physiological function in estrogen-sensitive cells.

GPER and ER-α36 are similarly regulated by estrogen and icariin

In addition to ER-α36, we have demonstrated that GPER is expressed in bone tissues (tibia head) of SD rats and osteoblastic cells (MC3T3-E1 and MG-63 cells). OVX increased GPER protein expression which could be reversed by the treatment of icariin and E_2. *In vitro* study showed that both icariin and E_2 could suppress the expression of GPER in both MC3T3-E1 cells and MG-63 cells upon treatment for 24 hours. The results indicate that GPER and ER-α66 are also inversely regulated in a way similar to ER-α36. These results are consistent with other studies that introducing GPER could induce endogenous ER-α36 in GPER non-expressing cells HEK293 cells, while knockdown of GPER could lead to downregulation of ER-α36 in breast cancer SK-BR-3 cells (X. T. Zhang et al., 2011). Moreover, GPER-specific agonist was found to recruit ER-α36 in activating the phosphorylation of MAPK/ERK1/2 in SK-BR-3 cells (X. T. Zhang et al., 2011). These results shed light on understanding the crosstalk amongst ERs and suggested GPER seems to be the positive regulator of ER-α36 expression in bone upon icariin and E_2 treatment.

Regulation of ER-α36 expression by E_2 or icariin is GPER-dependent and ER-α66-independent

We next, evaluated the crosstalk among ER-α66, ER-α36, and GPER using ER-α66-negative HEK293 cells. We have successfully established ER-α66-knockin, ER-α36-overexpressed or GPER-overexpressed in HEK293 cells. Notably, overexpression of GPER increased both GPER and ER-α36 expression, suggesting that GPER could induce ER-α36 expression. It is further confirmed by dual luciferase assay that GPER overexpression could stimulate ER-α36

promoter activity expression. Indeed, such observation has been previously reported (Han et al., 2015) .

E_2 and icariin could downregulate the expression of both GPER and ER-α36 in HEK293 cells with GPER overexpression (Lianguo Kang et al., 2010). These results suggest that E_2 and icariin downregulate ER-α36 probably via the suppression of GPER expression. Previously, other group suggested that GPER could physically interact with ER-α36 on the cell membrane as a coregulator mediating anti-inflammatory action of E_2 in human primary monocytes (Pelekanou et al., 2016). Thus, the simultaneous regulation of their expressions might give a hint on their collaborative role in bone remodelling upon icariin and E_2 treatment. Further study is need to investigate the localization of ER-α36 and GPER in response to icariin and E_2 during bone remodelling.

Knockin of ER-α66, on the other hand, only promoted ER-α66 protein expression in HEK293 cells. The treatment of E_2 and icariin did not alter the expression of ER-α66 in HEK293 cells. In contrary, overexpression of ER-α36 only promoted ER-α36 protein expression in HEK293 cells and that could be suppressed by the treatment of E_2 and icariin. The co-transfection of ER-α66 and ER-α36 expression vector could induce the expression of both ERs protein. Interestingly, E_2 and icariin treatment could reverse the overexpression of both ER-α66 and ER-α 36 in HEK293 cells. Taken together, both icariin and E_2 downregulate ER-α66 and ER-α36 expression. The differential regulation of ER-α66 and ER-α36 in ER-α66-negative HEK293 cells were also found in MC3T3-E1 cells and MG-63 cells, in which ER-α 36 and ER-α 66 are oppositely regulated in response to E_2 and icariin. Further study is needed to understand the functional meaning of ERs' crosstalk in bone protection of icariin and E_2 in osteoblast.

In summary, the expression of GPER seems to be associated with ER-α36 expression in HEK293 cells with or without treatment with E_2 or icariin. Icariin treatment could not regulate ER-α36 expression when GPER is absent. Also, the knock-in of ER-α66 did not affect ER-α36 expression. Therefore, the expression of ER-α36 and GPER is likely to be associated with the expression of GPER but not ER-α66 in HEK293 cells (Figure 3.7a).This chapter provided new insights regarding the regulation of GPER, ER-α36, and ER-α 66 expression in osteoblasts and their differential responses to E_2 and icariin (Figure 3.7b). ER-α 36 and GPER might work together as coregulator and act differently from ER-α 66 in mediating the actions of icariin. These novel estrogen receptors could be possible candidates for mediating the ligand-independent pathway activated by icariin in bone. The roles of these ERs in bone protection and activation of signaling transduction by icariin in bone will be illustrated in chapter 4.

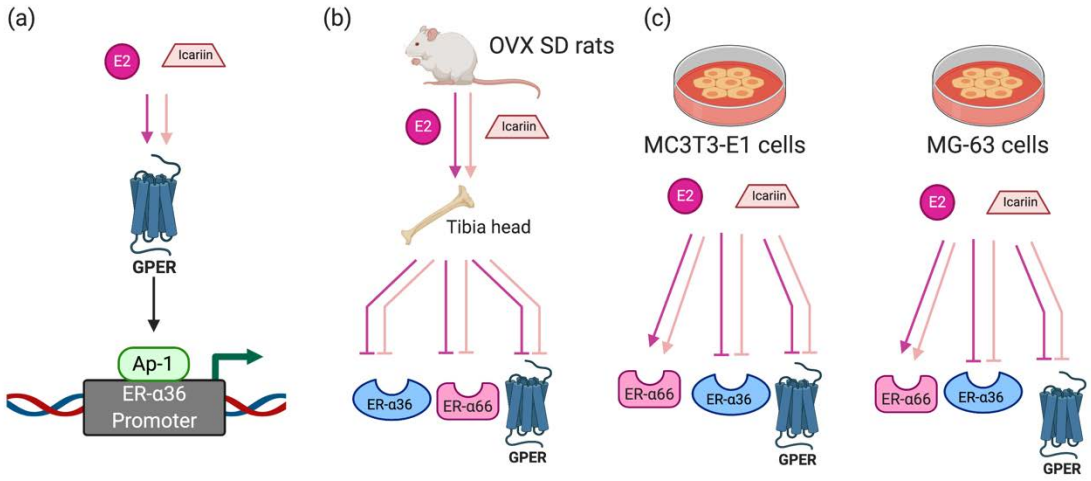


Figure 3.7 Schematic mechanisms illustrating the tissue responsiveness via ERs in response to E_2 and icariin in bone.

The protein expression of ER-α36 regulated by E_2 and icariin is ER-α66-independent, but GPER-dependent (a). *In vivo*, E_2 and icariin reversed OVX-induced protein expression of ER-α66, ER-α36 and GPER in tibia heads (b). Both E_2 and icariin induced ER-α66 protein expressions while suppressed GPER protein expressions *in vitro* (c).

Chapter 4. Characterization of the role of GPER and ER-α36 in bone protective effects of icariin

4.1 Introduction

The bone protective effects of icariin, a major flavonoid in a clinically used Chinese herb *Herba Epimedii* for treatment of bone disease, were well characterized. Icariin was shown to promote bone formation by stimulation of osteoblastic differentiation, suppression of adipocytes differentiation in bone marrow stromal cells (BMSCs), and inhibition of bone resorption activities of osteoclasts (M. S. Wong & Zhang, 2013). Results from our team indicated that icariin acted like a pathway-specific Selective Estrogen Receptor Modulator (SERM) exerting bone protective effects via differential activation of membrane initiated, but not nuclear, ER signaling pathway (Zhou et al., 2019). Our earlier studies also showed that icariin exerted estrogen-like protective effects on bone in OVX mice (Zhou et al., 2019) and modulated the estrogenic bone remodelling by regulating the expression of estrogen-sensitive bone turnover markers, osteocalcin (OCN), osteoprotegerin (OPG), alkaline phosphatase(ALP), and receptor activator of nuclear factor kappa-B ligand (RANKL) in rat osteoblast-like UMR 106 cells (Mok et al., 2010). Moreover, *in vitro* studies suggested that icariin could elicit ER-dependent, ligand-independent rapid estrogenic signaling, including MAPK/ERK/ER-α(Ser118) and PI3K/Akt/ER-α(Ser167) in murine preosteoblast MC3T3-E1 cells and UMR 106 cells (Ho et al., 2018).

In chapter 3, we first identified the recruitment of novel membrane estrogen receptors, estrogen receptor α 36 (ER-α36) and G-protein coupled estrogen receptor (GPER), by estradiol (E_2) and icariin, beside the classical ER-α66, in bone. Previously, ER-α36 was reported to be a novel isoform of classical ER-α66 that is transcribed from a previously undiscovered promoter in intron 1 of ER-α66. The lack of AF-1 and AF-2 domain of ER-α66, but presence of ERK binding site in domain D in ER-α36 enable it to preferably activate estrogenic signaling. Upon E_2 activation, ER-α36 rapidly dissociated from EGFR or caveolin-1 (Lin et al., 2010) and form signaling complex with downstream effectors including Src, Shc, insulin-like growth factor 1 receptor (IGF-1R) within minutes of activation (Omarjee et al., 2017). Also, the conserved ERK binding site in the domain D of ER-α36 allows it to stimulate the MAPK/ERK pathway in ER-α66-negative breast cancer HBL100 cells (Omarjee et al., 2017). Clinically, the expression of ER-α36 was reported to positively correlate with bone mineral density (BMD) and negatively associate with biochemical bone markers in Chinese postmenopausal women (Xie et al., 2011). Thus, it is anticipated that membrane ER-α36 might involve in the bone protection of icariin and E_2 by mediating nongenomic estrogen signaling.

GPER is a seven-transmembrane-spanning estrogen receptor that is known to mediate rapid estrogenic signaling. Upon ligand binding and intracellular trafficking from rough endometrium reticulum to the cell membrane (Gaudet et al., 2015), it could trigger intracellular cAMP production and activation of estrogenic signaling, PI3K/Akt and ERK1/2 pathways (Prossnitz & Barton, 2011). It was also found to negatively regulate osteogenic-differentiation in murine bone marrow mesenchymal stem cells (J. Zhong et al., 2019).

Recent studies suggest that ER-α36 might be the downstream effort or collaborator of GPER. GPER physically interacts with ER-α36 (Pelekanou et al., 2016) and could positively regulate ER-α36 expression(Han et al., 2015). G-1, previously thought to be a GPER-specific agonist, failed to activate GPER-mediated rapid estrogenic pathway in ER-α36-negative human embryotic kidney 293 (HEK293) cells (Lianguo Kang et al., 2010). Thus, GPER might also involve in the nongenomic estrogenic bone protection induced by icariin via ER-α36/GPER crosstalk. Indeed, we have suggested in chapter 3 that icariin and E_2 could interrupt the crosstalk among ER-α36, GPER, and classical ER-α66. Previous studies proposed that ER-α36 shares a similar DNA binding domain with ER-α66 which could inhibit the ER-α66-stimulated genomic regulation by competing for the binding sites. Also, intact dimerization domain in ER-α36 favours the formation of ER-α36/ER-α66 heterodimer which could prevent the binding of ER-α66 to transcriptional factor in the nucleus by retaining ER-α66 in the cytoplasm (Z. Y. Wang & Yin, 2015). Thus, it is worthy to study if the interruption of ERs crosstalk would affect ER-α66-mediated gene expression of bone marker upon icariin treatment.

In this chapter , the involvement of ER-α36 or GPER in the estrogen-like bone protective effect of icariin via ER-α66-mediated rapid signaling are investigated (Figure 4.1). We utilized rat BMSCs (rBMSCs) to study the ER cellular translocation and formation of signaling complexes (Shc, src) which could reveal the activation of ERs during rapid signaling. Also, MC3T3-E1 cells and MG-63 cells were transiently transfected with ER-α36 expression vector or GPER siRNA or pre-treatment with G15, a GPER-specific antagonist. Taking advantage of these cell models, the effect of ER-α36 or GPER on icariin- or E_2- induced osteoblastogenesis, including cell proliferation; ER-α66-mediated bone marker expression as well as signaling transduction, could be studied. It is hoped that this study could increase our understanding of the mechanism of actions by which icariin elicit membrane-initiated ligand-independent estrogenic signaling in bone cells and provide the mechanistic basis for developing a new generation of pathway selective agent for prevention and treatment of osteoporosis.

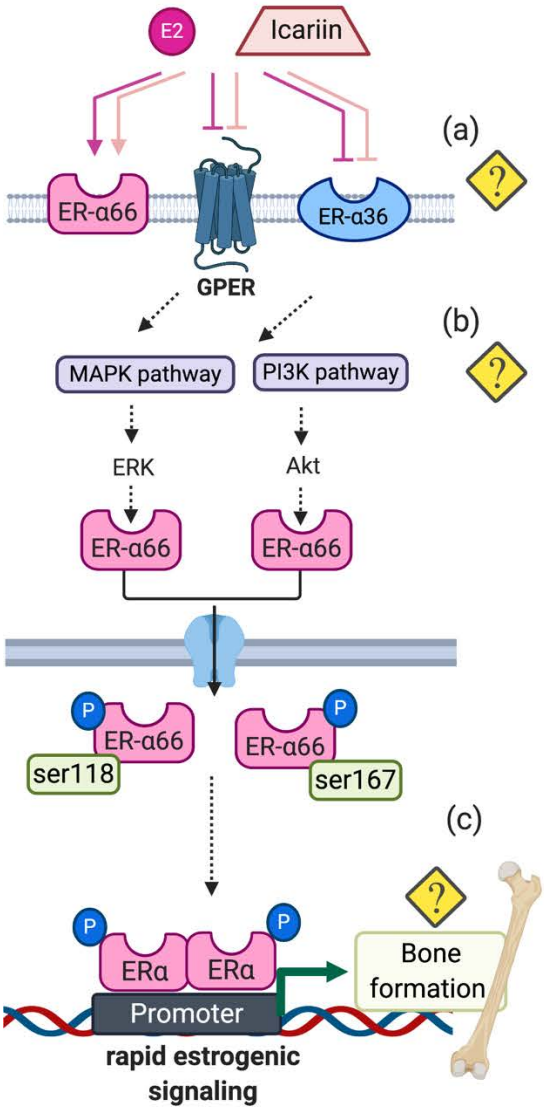


Figure 4.1 Overview of Chapter 4.

The protein regulations of ER-α36, ER-α66, and GPER have been revealed in chapter 3 as shown with red and blue indicators. The signal transduction and the formation of signaling complexes (a) and activation of estrogen signaling via these ERs induced by icariin (b) as well as endpoint study of osteoblastogenesis mediated by these ERs(c) will be illustrated in this chapter.

4.2 Methodology

4.2.1 Experimental design

To systematically investigate the roles of ER-α36 and GPER in mediating the bone protective effects of icariin, mouse pre-osteoblast MC3T3-E1 cells, human osteoblast-like MG-63 cells, and osteoblasts differentiated from rat bone marrow stromal cells (rBMSCs) were employed. MC3T3-E1 cells and MG-63 cells were used to investigate the role of ER-α36 and GPER in the action of icariin in the pre-osteoblastic and mature osteoblastic stage. First, overexpression of ER-α36, GPER knockdown, or pre-treatment of G15 (a GPER specific antagonist) was performed in these cells before treatment with icariin. Bone protective effects of icariin in cells with transfection was assessed by MTS assay as well as mRNA expressions of bone markers. The effects on signal transduction were studied by measuring phosphorylation of signaling molecules in Akt and MAPK pathways in these two cell lines and the translocation and formation of signaling complexes among ERs in response to icariin were studied in ERs-rich osteoblast differentiated from rBMSCs.

4.2.2 Cell culture

MC3T3-E1 cells and MG-63 cells were as cultured as described in chapter 3. Cells were seeded at a quantity of approximately 1×10^4 cells/well or 8×10^3 cells/well into 96-well flat-bottomed cell culture plates, respectively. Besides, rBMSCs were collected from 3-6-month old female SD rats followed by osteoblastic differentiation in low-glucose DMEM supplemented with 1% penicillin and streptomycin (Life Technologies, Rockville, MD, United States, Cat# 15140-122), 1% Fungizone (Thermo Fisher Scientific, Rockford, IL, United States, Cat#15290026), 100μM L-ascorbic acid, 10nM dexamethasone, and 10% FBS for 21 days in a humidified environment at 5 % CO_2 and 37 °C. The medium was replaced every three days. Differentiated cells for experiments were seeded at a quantity of approximately 12×10^4 cells/well into 6-well flat-bottomed cell culture plates, respectively.

4.2.3 Transformation and transfection

To investigate the involvement of ER-α36 and GPER in the actions of icariin in bone, knockin, knockdown, or overexpression of ERs were employed. MG-63 cells, MC3T3-E1 cells, and HEK293 cells were seeded in a 6-well plate for 24 hours with standard culture medium before replacing the medium with phenol-red free (PRF) DMEM or αMEM without supplementation of FBS and antibiotics. After 24 hours, the cells were transfected with 1μg CMV-36, 1μg CMV-HA, 1μg CMV-GPER, 0.4μg ER-α66 plasmid, 2.5pmol mus GPER siRNA (Life technology, Cat# 4370771), 2.5 pmol human GPER siRNA(Life technology, Cat#4392420) or

10pmol negative control siRNA (Life technology, Cat# 4390843) by 4μl/well Lipofectamine ™ 2000 reagent (Invitrogen, Cat#11668-019) in PRF medium without antibiotics and FBS for 24 hours. After 24 hours, the medium was removed and replaced with PRF-medium with antibiotics and FBS and cultured for 24 hours allowing expression of the vector of the breakdown of target mRNA before cell treatment. The transfection efficiencies were examined by Western blotting and real-time PCR.

4.2.4 **MTS Assay**

Icariin at various concentrations (10^{-12} to 10^{-6}M), E_2 at 10^{-8}M, or its vehicle was added to the cells for 24 hours, followed by the determination of cell proliferation using MTS assay (Promega, Ann Arbor, MI, Cat# G3582). The absorbance was recorded in UFLUOstar Omega (BMG LABTECH, USA) at 490 nm with MTS working solution as blank. Results were expressed at the ratio relative to control groups

4.2.5 **Real-time PCR**

mRNA expression of different bone markers was examined, including osteoprotegerin (OPG) and osteocalcin (OCN) for bone formation, nuclear factor kappa-B ligand(RANKL) for bone resorption and alkaline phosphatase(ALP) for bone differentiation using specific primers listed in Table 5.1. Cells were seeded on a 6-well plate and treated with optimal dosages of icariin as determined by MTS and ALP assays for 24 hours in the PRF medium. Details of mRNA extraction, reserve transcription, as well as real-time PCR, have been illustrated in chapter 3.

4.2.6 **Western blotting**

To study the rapid signaling activation upon icariin treatment, protein expression of signaling molecules, including p-Akt, p-ERK, p-ER-α(Ser167) and (Ser118), were studied. Cells were seeded in a 6-well plate for 24 hours and treated with icariin at optimal doses for 10 minutes. Proteins were extracted, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis. Briefly, the transferred proteins on PVDF membranes were blocked with % blotting-grade blocker (Bio-Rad, Cat#1706404) for 2 hours and subsequently probed overnight at 4^{o}C with the following primary antibodies: mouse anti-phospho-ERα at Ser118 (1:2000, Upstate), mouse anti-phospho-ERα at Ser167 (1:2000, Upstate), rabbit anti-phospho-Akt at Ser473 (1:1000, Cell Signaling), rabbit anti-Akt1/2/3 (1:1000, Santa Cruz), mouse anti-phospho-ERK (1:1000, Santa Cruz), rabbit anti-ERK1/2 (1:1000, Cell Signaling), mouse anti-ER-α66 (1:1000, Santa Cruz, Cat#Sc-544)or mouse anti-β actin (1:2000, Abcam). The membranes were then washed three times with Tris Buffered Saline Buffer with Tween 20 (TBST) for 30 minutes in total, followed by incubation

with anti-rabbit (1:1000, Santa Cruz, Cat# Sc-2004) or anti-mouse (1:1000, Cell Signaling Technology, Cat#7076) IgG-HRP-conjugated secondary antibodies for 1 hour. Detection of band intensities were illustrated in chapter 3.

4.2.7 **Immunostaining**

To visualize the translocation of ERs in rapid estrogen-like response upon icariin treatment, rBMSCs treated with icariin for 10 minutes were fixed immediately with 4% paraformaldehyde and were permeabilized with 0.5% Triton X-100 for 1 min. Upon blocking with 1% BSA for 45 min, the cells were incubated with specific primary antibodies mouse anti-ER-α66 (1:1000, Santa Cruz, Cat#Sc-544), rabbit anti-GPER (1:1000, Abcam, Cat#ab39742) or mouse anti-ER-α36 provided by Professor Wang Zhao-yi from Beijing Shenogen Pharma Group Ltd. (1:1000) overnight at 4°C. The primary antibodies were probed with either Alexa Fluor 488-conjugated anti-rabbit antibody or Alexa Fluor 594-conjugated anti-mouse antibody for one hour at room temperature. DAPI counter-staining was applied for determining the intensity of a single cell. Fluorescence images were captured at mid-plane of cells (oil objective; magnification: 600X) by the Leica TCS SPE DMi8 confocal microscope (Leica Microsystems, Wetzlar, Germany). The overall intensities of the fluorescent signals of a single cell were also quantified using the corresponding Leica Microsystems software station (LAS AF, Leica Microsystems, Germany).

4.2.8 **Immunoprecipitation**

To assess the formation of signaling complexes during estrogen-like rapid response upon icariin treatment, osteoblasts were treated with icariin for 10 minutes, followed by lysis with Nonidet P-40 buffer. Protein lysate was precipitated with the corresponding antibodies overnight. The antibody/antigen complex was pulled out by the addition of protein A-Sepharose slurry. Precipitated proteins were resuspended and subjected to immunoblotting as described above. The antigen-antibody complexes were then detected with enhanced chemiluminescence reagent and visualized by Azure c600 (Azure Biosystems, USA).

4.2.9 **Statistical analysis**

Inter-group difference in both *in vitro* and *in vivo* study were determined by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. All graphs in this study were plotted by using GraphPad Prism Version 7.0.

Table 4.1 Primer sequences for MG-63 cells and MC3T3-E1 cells.

	MG-63 cells	MC3T3-E1 cells
Gene	Sequence	Sequence
ALP	TTTATAAGGCGGCGGGGGTG AGCCCAGAGATGCAATCGAC	ACTTGGTGGTCACAGCAGTTG TGGAGACGCCCATACCATCT
GAPDH	AGGGCTGCTTTTAACTCTGGT CCCCACTTGATTTTGGAGGGA	TCAGGAGAGTGTTTCCTCGTC GGCCTCACCCCATTTGATGT
OCN	AGCTCCCAACCACAATATCCT TTATACCCTCTGGGCTGTGC	ATGAGGACCCTCTCTCTGCT CCGTAGATGCGTTTGTAGGC
OPG	ACAGCAAAGTGGAAGACCGT CCTTCCTTGCATTCGCACAC	TGAGAGAACGAGAAAGACCTGC CGGATTGAACCTGATTCCCTAT
RANKL	GGGGAAAACTTGCAGCTAAGG AATTTGCGGCACTTGTGGAA	TCCTGAGACTCCATGAAAACGCAG GCCACATCCAACCATGAGCCTTC

4.3 Results

4.3.1 Establishment of osteoblastic cell models

To investigate the role of ER-α36 and GPER in the bone protective effects of icariin, ER-α36 overexpressed and GPER-knockdown cells were established in osteoblastic MC3T3-E1 and MG-63 cell lines. Our results (Figure 4.2) showed that the protein expressions of GPER in MC3T3-E1 cells were significantly knocked down when cells were transfected with 10nM, 25nM, and 40nM mouse GPER-specific siRNA for 48 hours (P <0.05 vs. NT siRNA). The results indicated that the best concentration of siRNA causing 50% knocking down efficiency was 40nM. The knockdown of GPER did not alter the protein expression of ER-α66 and ER-α36 in MC3T3-E1 cells. On the other hand, the expression of ER-α36 protein was enhanced by 2-fold by transfection with 5mg/ml ER-α36 expression vector in MC3T3-E1 cells, without altering the expression of ER-α66 (P <0.05 vs. CMV). MC3T3-E1 cells transfected with 5mg/ml ER-α36 expression vector have better overexpression of ER-α36 than those transfected with 2mg/ml vector. As for MG-63 cell, GPER protein expressions were significantly reduced by half when MG-63 cells were transfected with 25 nM human GPER-specific siRNA (P <0.05 vs. NT siRNA), without changes in the protein expression of ER-α36 and ER-α66. Last, the protein expression of ER-α36 was dramatically upregulated in MG-63 cells transfected with 1, 2 or 5mg/ml ER-α36 expression vector (P <0.05 vs. CMV), whereas the best transfection efficiency was observed when cells were transfected with 5mg/ml ER-α36 expression vector.

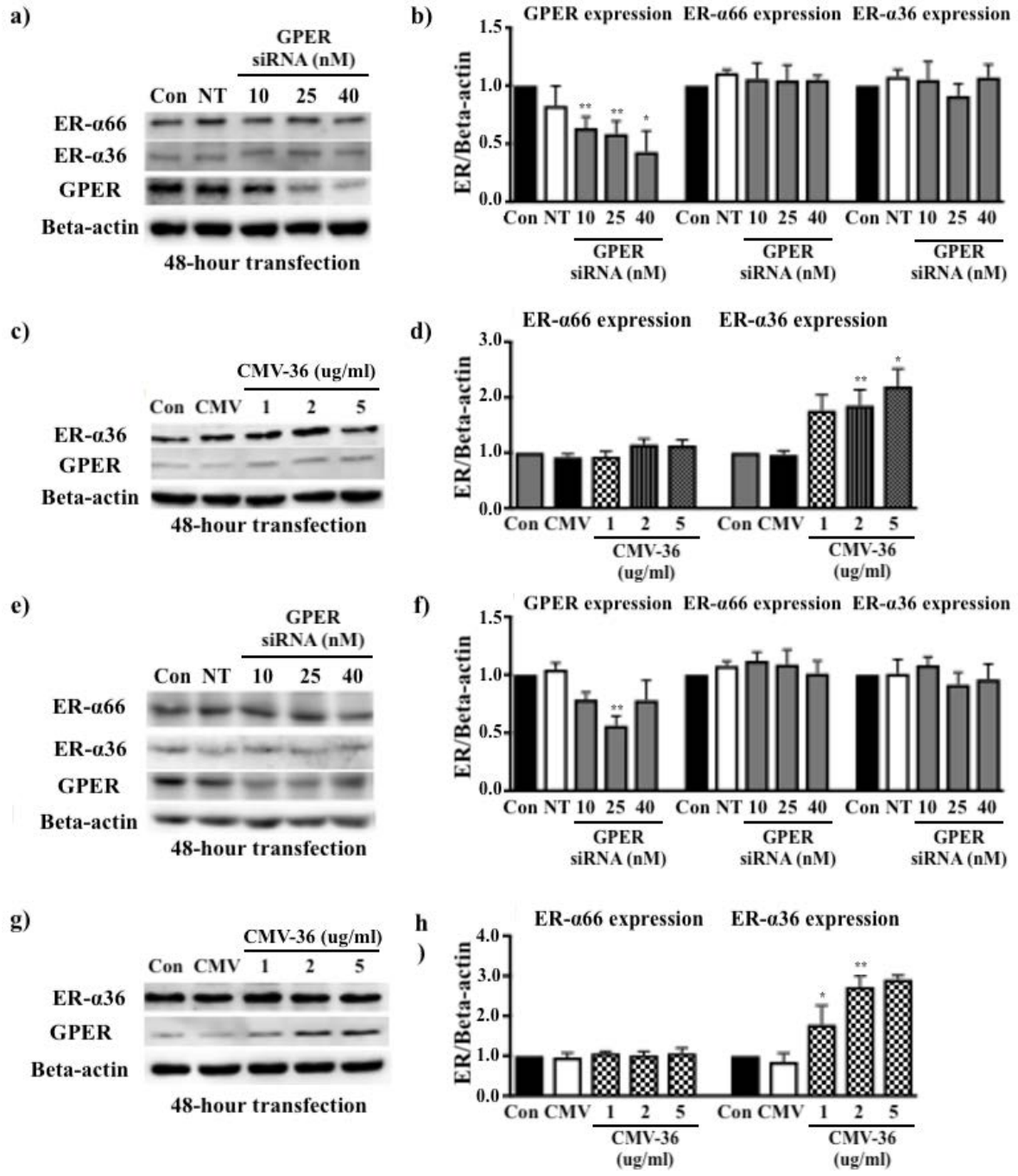


Figure 4.2 Establishing ER-α36 overexpression or GPER knockdown cell models.

MC3T3-E1 cells and MG-63 cells were transfected with control plasmids (CMV), ER-α36 expression plasmids (CMV-36), non-targeting siRNA (NT) or GPER siRNA for 48 hours. The expression of ER-α66, ER-α36, and GPER in MC3T3-E1 cells (a-d) and MG-63 cells (e-h) were analyzed by western blotting. The figures are the representatives of three independent experiments in both cells. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control.

4.3.2 Characterization of the role of GPER in bone protective effect of icariin

4.3.2.1 Effect of GPER antagonist and siRNA on cell viability in icariin-treated osteoblasts

The results (Figure 4.3) showed that icariin, acted like E_2, increased cell viability of MC3T3-E1 cells and MG-63 cells at the dosages from 10^{-8}M to 10^{-6}M and from 10^{-10}M to 10^{-8}M, respectively. These dosages were chosen to be the optimal doses. G15 pre-treatment for 20 minutes did not affect the cell viability of both MC3T3-E1 cells and MG-63 cells at their basal state when compared to the control group. However, G15 pre-treatment significantly enhanced the induction by icariin on cell viability at 10^{-7}M and 10^{-8}M in MC3T3-E1 cells and MG-63 cells, respectively (P <0.05 vs. treatment group without G15). NT siRNA transfection did not affect the cell viability of both cells when compared to the control group. GPER siRNA transfection significantly enhanced the induction of cell viability by icariin at 10^{-7}M and 10^{-8}M in MC3T3-E1 and MG-63 cells, respectively (P <0.05 vs. treatment group transfected with NT siRNA).

4.3.2.2 Effect of GPER antagonist on mRNA expression of bone markers in icariin-treated osteoblasts

Our results (Figure 4.4) showed that icariin at 10^{-6}M, acted like E_2, significantly increased the mRNA expression of OCN by 1.2-fold, OPG by 1.2 fold, and OPG/RANKL ratio by 2-fold while reduced RANKL mRNA expression by 0.6-fold in MC3T3-E1 cells (P <0.05 vs. Control). ALP mRNA expression was not altered in MC3T3-E1 cells upon treatment with icariin. Moreover, pre-treatment of MC3T3-E1 cells with G15 further increased OPG mRNA induced by 10^{-7}M icariin (P <0.05 vs. Treatment group without G15 pre-treatment). Similarly, E_2 and icariin increased the mRNA expression of OCN by 1.5-fold, OPG by 1.2-fold and OPG/RANKL ratio by 1.5-fold, and decreased RANKL mRNA expression by 0.7-fold in MG-63 cells (P <0.05 vs. Control). Pre-treatment of MG-63 cells with G15 significantly enhanced the stimulation of OCN and OPG mRNA expression by icariin in MG-63 cells (P <0.05 vs. Treatment group without G15 pre-treatment).

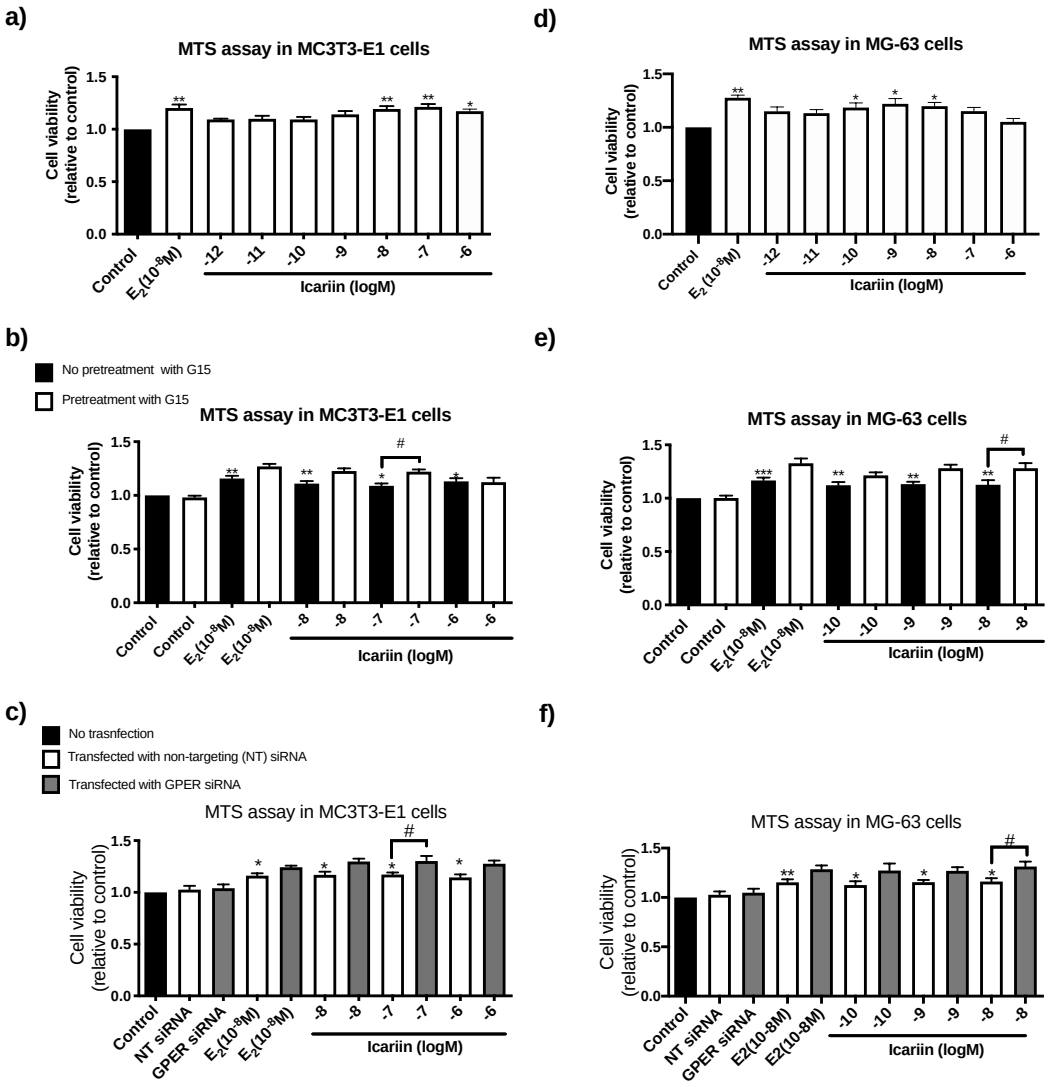


Figure 4.3 The effect of icariin on cell viability in osteoblasts with GPER knockdown or G15 pre-treatment.

MC3T3-E1 cells (a) and MG-63 cells (b) were treated with 10^{-12}M to 10^{-6}M icariin for 24 hours to determine the optimal dosages. MC3T3-E1 cells (c-d) and MG-63 cells (e-f) were then transfected with non-targeting siRNA (NT), GPER siRNA at desired dosages or pre-treated with G15 (10^{-6}M) before the 24-hour vehicle, E_2 or icariin treatment. Cell viability was assessed by MTS assay. The figures are the representatives of three independent experiments in MC3T3-E1 and MG-63 cells. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control (a, b, d and e) or NT siRNA (c and f); # $P<0.05$ vs. Treatment group transfected with NT siRNA(c and f) or pre-treated with G15 (d and e).

4.3.2.3 **Effect of GPER siRNA on mRNA expression of bone markers in icariin treated osteoblasts**

The results in figure 4.5 showed that icariin at 10^{-8}M, acted like E_2, significantly increased the mRNA expression of OCN by 1.2-fold OPG by 1.5-fold and OPG/RANKL ratio by 2-fold while reduced RANKL mRNA expression by 0.6-fold in MC3T3-E1 cells (P <0.05 vs. NT siRNA). ALP mRNA expression was not altered by treatment with icariin in MC3T3-E1 cells. GPER knockdown by siRNA further boosted OPG mRNA induced by icariin (10^{-7}M) in MC3T3-E1 cells (P <0.05 vs. Treatment group transfected with the control vector).

Similarly, E_2 and icariin at 10^{-8}M significantly increased the mRNA expression of OCN by 1.5-fold, OPG by 1.2-fold and OPG/RANKL ratio by 2-fold, and reduced RANKL mRNA expression by 0.7-fold in MG-63 cells (P <0.05 vs. NT siRNA). GPER knockdown by siRNA further enhanced the stimulation by icariin on OPG mRNA expression in MG-63 cells (P <0.05 vs. Treatment group transfected with control vector).

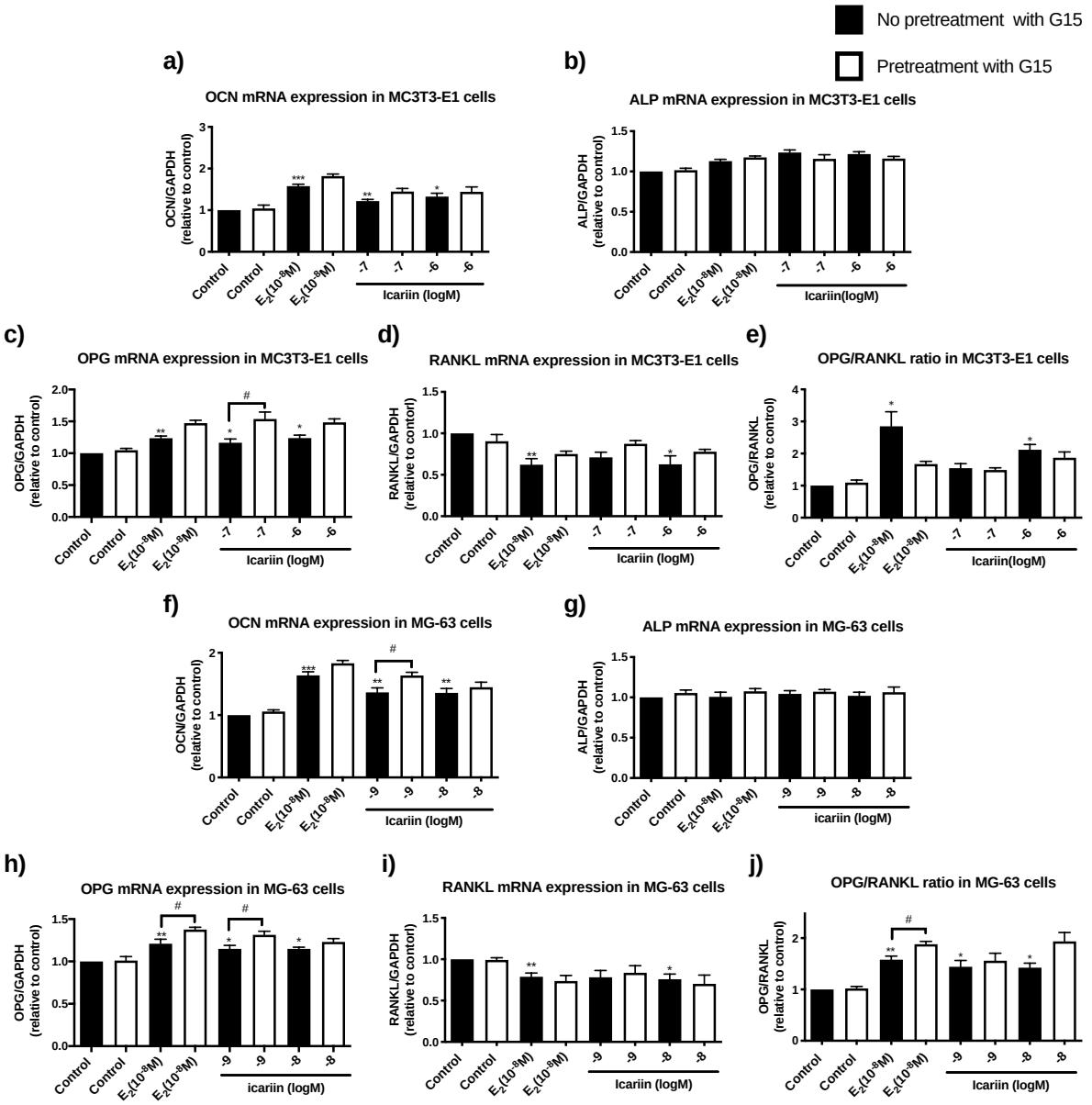


Figure 4.4 Effect of GPER antagonist on mRNA expressions of bone markers induced by icariin in osteoblasts.

MC3T3-E1 cells and MG-63 cells were pre-treated with GPER antagonist, G15 before the vehicle, E_2, or icariin treatment for 24 hours. mRNA was harvested by Trizol reagent. mRNA expressions of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio in MC3T3-E1 cells (a-e) and in MG-63 cells (f-j) were amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control. $^{\#}$ $P < 0.05$ vs. Treatment group transfected pre-treated with G15.

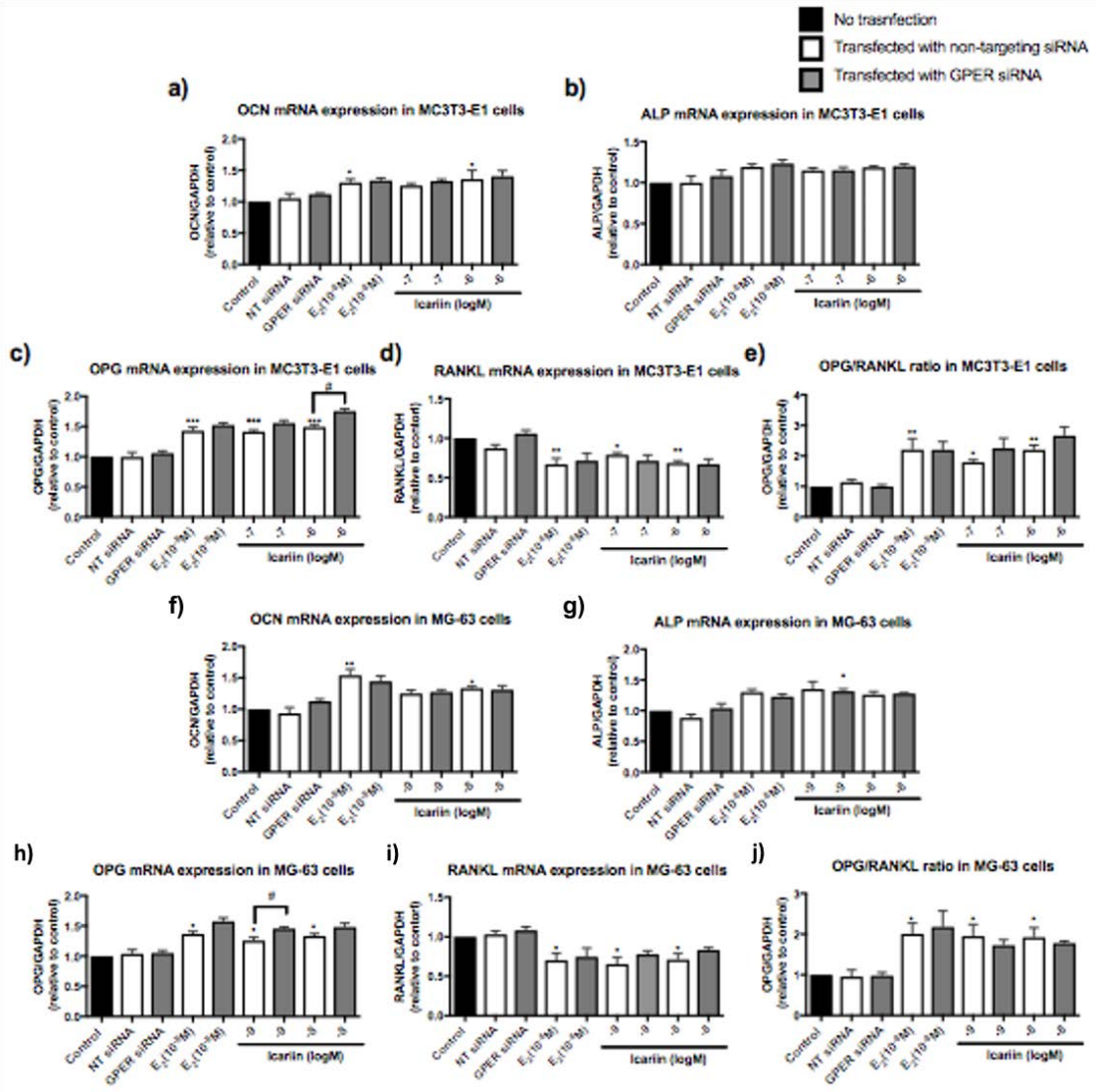


Figure 4.5 Effect of GPER knockdown on mRNA expressions of bone markers induced by icariin in osteoblasts.

MC3T3-E1 cells and MG-63 cells were transfected with GPER siRNA before the vehicle, E_2, or icariin treatment for 24 hours. mRNA was harvested by Trizol reagent. mRNA expression of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio in MC3T3-E1 cells (a-e) and in MG-63 cells (f-j) was amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. NT siRNA. $^{\#}$ $P < 0.05$ vs. Treatment group transfected with NT siRNA.

4.3.2.4 **Effect of GPER antagonist and siRNA on estrogenic signaling in icariin-treated osteoblasts**

Our results (Figure 4.6) demonstrated that E_2 (10^{-8}M) and icariin (10^{-6}M) rapidly induced the phosphorylation of Akt, ERK, ER-α(Ser167), and (Ser118) in MC3T3-E1 cells (P <0.05 vs. Control) in 10 minutes. Pre-treatment with G15 further enhanced E_2-induced phosphorylation of Akt and ER-α (Ser167) and icariin-induced phosphorylation of ERK and ER-α66 (Ser118) within 10 minutes in MC3T3-E1 cells (P <0.05 vs. treatment group without G15 pre-treatment). Similarly, E_2 (10^{-8}M) and icariin (10^{-8}M) rapidly increased the phosphorylation of Akt, ERK, ER-α (Ser167), and (Ser118) in MG-63 cells (P <0.05 vs. Control). Pre-treatment with G15 further enhanced E_2-induced phosphorylation of Akt, ERK, and ER-α (Ser167) within 10 minutes but did not affect icariin-induced phosphorylation of these signaling molecules (P <0.05 vs. treatment group without G15 pre-treatment).

4.3.2.5 **Effect of GPER siRNA on estrogenic signaling in icariin-treated osteoblasts**

In figure 4.7, knockdown of GPER in MC3T3-E1 cells did not alter E_2-induced phosphorylation of Akt, ERK, ER-α (Ser167), and (Ser118) while GPER knockdown only further (P <0.05 vs. treatment group without G15 pre-treatment) enhanced phosphorylation of Akt in MG-63 cells. Moreover, our results demonstrated that GPER knockdown further enhanced icariin-induced phosphorylation of Akt and ER-α (Ser167) within 10 minutes of treatment in MC3T3-E1 cells (P <0.05 vs. treatment group without G15 pre-treatment). Similarly, GPER knockdown further enhanced icariin-induced phosphorylation of Akt, but not other signaling molecules, within 10 minutes of treatment in MG-63 cells (P <0.05 vs. treatment group without G15 pre-treatment).

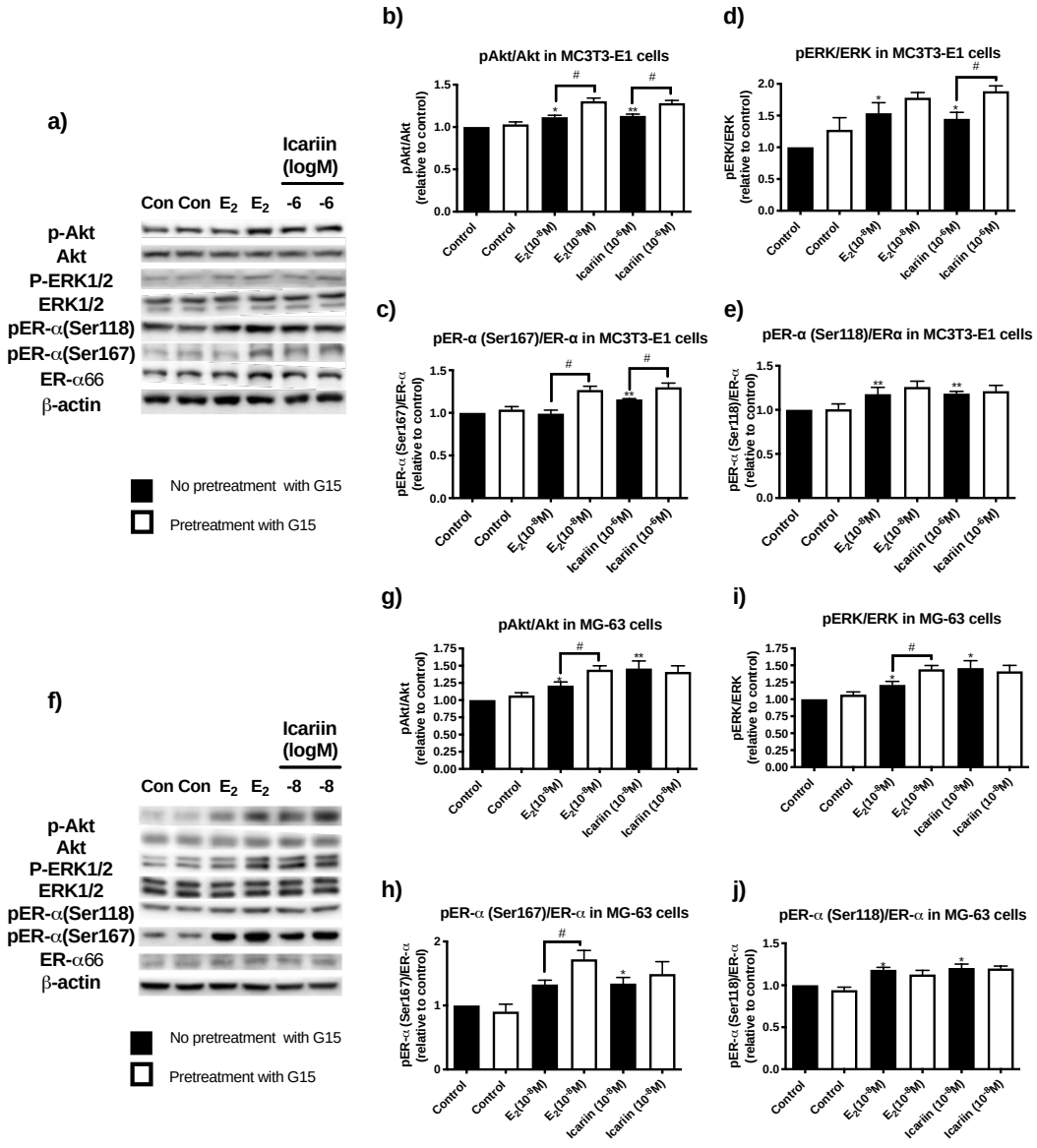


Figure 4.6 The effects of G15 pre-treatment on rapid signaling activated by icariin in osteoblasts.

MC3T3-E1 cells (a-c) and MG-63 cells (f-j) were pre-treated with G15(10^{-6}M) for 20 minutes before treatment with vehicle, E_2, or icariin for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER (Ser167), pER (Ser118), and ER-α66 were detected by western blotting in both cells. The protein expression level was shown as the ratio of the target protein to β-actin. The figures are the representatives of three independent experiments in MC3T3-E1 cells and MG-63 cells. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}$ $p<0.05$ vs. Treatment group without G15 pre-treatment.

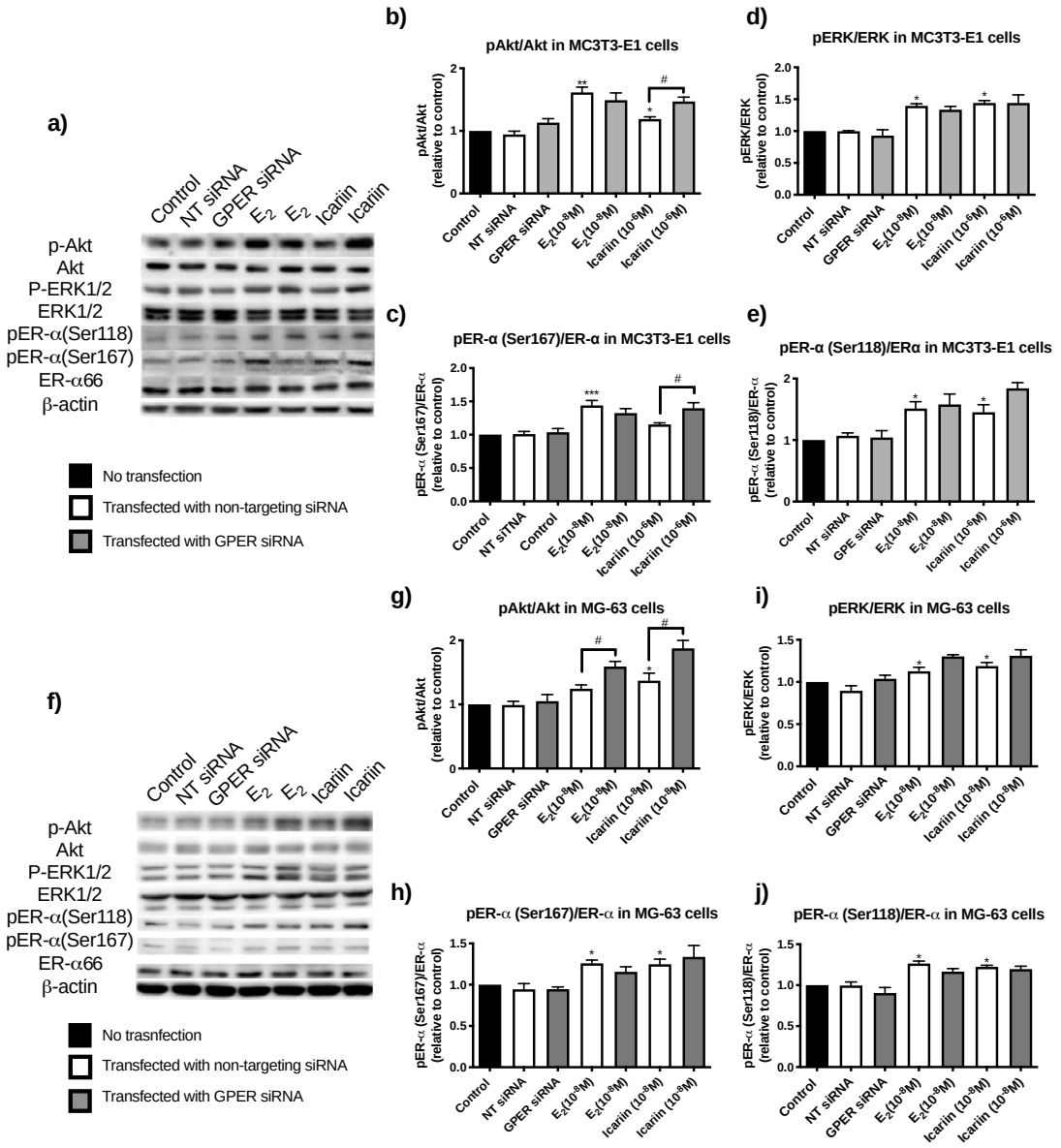


Figure 4.7 The effect of GPER knockdown on rapid ER signaling activated by icariin in osteoblasts.

MC3T3-E1 cells (a-e) and MG-63 (f-j) cells were transfected with non-targeting siRNA(NT) and GPER siRNA at desired dosages before treatment with vehicle, E_2, or icariin for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER-α (Ser167), pER-α (Ser118), and ER-α66 were studied by western blotting. The figures are the representatives of three independent experiments. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. NT siRNA; $^{\#}$ $p<0.05$ vs. Treatment group transfected with NT siRNA.

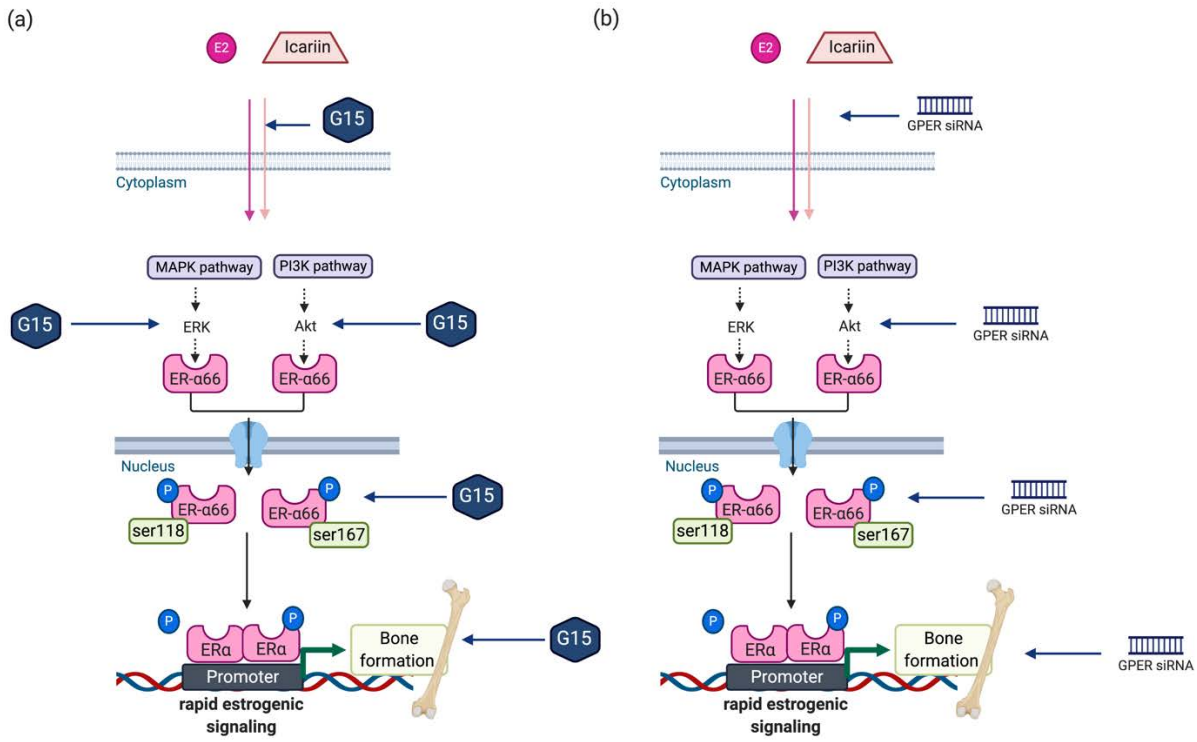


Figure 4.8 Summary of the GPER role in icariin action in bone.

Blocking of GPER with G15 (a) and GPER knockdown (b) could further enhance the icariin-induced stimulatory effect on cell viability, mRNA expression of bone formation markers, OPG, as well as activation of the AKT pathway via ER-α66 at ser a167. Only G15 pre-treatment could further activate the ERK pathway in osteoblasts upon icariin treatment.

4.3.3 Characterization of the role of ER-α36 in bone protective effect of icariin

4.3.3.1 Effect of ER-α36 overexpression on cell viability in icariin treated osteoblasts

As expected (Figure 4.9), E_2 at 10^{-8}M and icariin significantly (P <0.05 vs. Control) increased cell viability by 1.2-fold in MC3T3-E1 (10^{-8}M-10^{-6}M) and MG-63 cells (10^{-10}M-10^{-8}M). Overexpression of ER-α36 suppressed the E_2- or icariin-induced cell viability 10^{-9}M and 10^{-8}M in MG-63 cells (P <0.05 vs. Treatment group transfected with CMV), while overexpression of ER-α36 in MC3T3-E1 cells tended to suppress the effects of icariin on cell viability. However, the effect did reach statistical significance.

4.3.3.2 Effect of ER-α36 overexpression on mRNA expression of bone markers in icariin treated osteoblasts

In Figure 4.10, overexpression of ER-α36 significantly supressed icariin-induced mRNA expression of ALP at 10^{-8}M icariin and OCN from 10^{-7}M to 10^{-8}M icariin while reversed the inhibitory effects of icariin at 10^{-7}M on RANKL mRNA in MC3T3-E1 cells (P <0.05 vs. treatment group transfected with CMV). Moreover, the mRNA expression of OPG and OPG/RANKL ratio appeared to be reduced when MC3T3-E1 cells were transfected with the ER-α36 expression vector, but the changes did not reach statistical significance. In addition, overexpression of ER-α36 in MG-63 cells reversed the stimulatory effects of E_2 or icariin on OPG mRNA expression and the inhibitory effects on RANKL mRNA expression (P <0.05 vs. treatment group transfected with CMV) which in turn reduced the OPG/RANKL ratio in MG-63 cells upon treatment with 24-hour icariin or E_2 (P <0.01 vs. treatment group transfected with control vector).

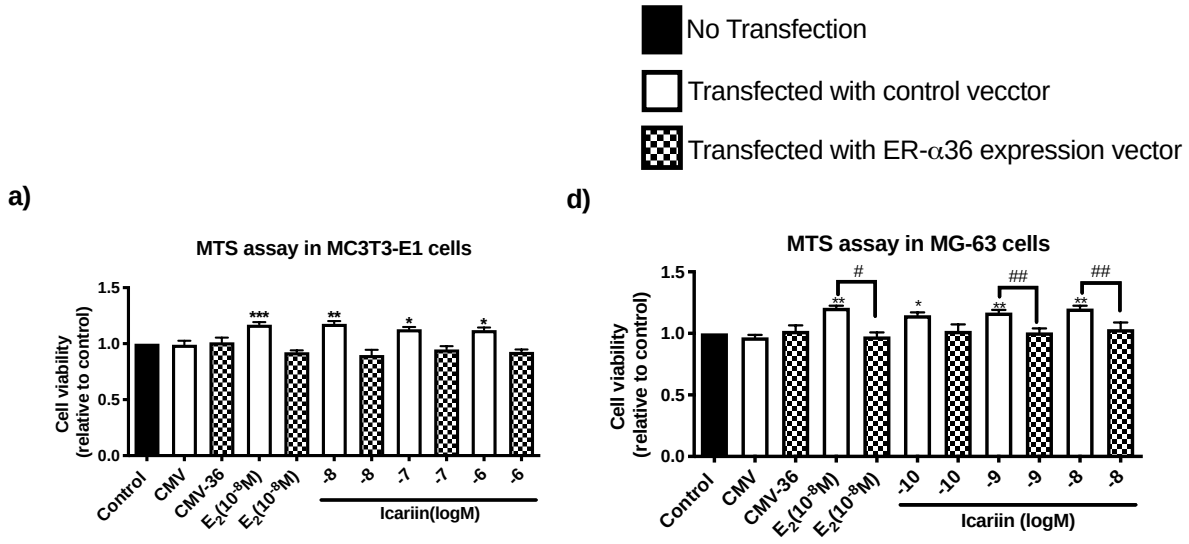


Figure 4.9 The effect of icariin on cell viability in osteoblasts overexpressed with ER-α36. MC3T3-E1 cells (a) and MG-63 cells (b) were transfected with CMV and CMV-36 plasmid at optimal doses before treatment with vehicle, E_2, or icariin for 24 hours. Cell viability was assessed by MTS assay. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Treatment group transfected with CMV.

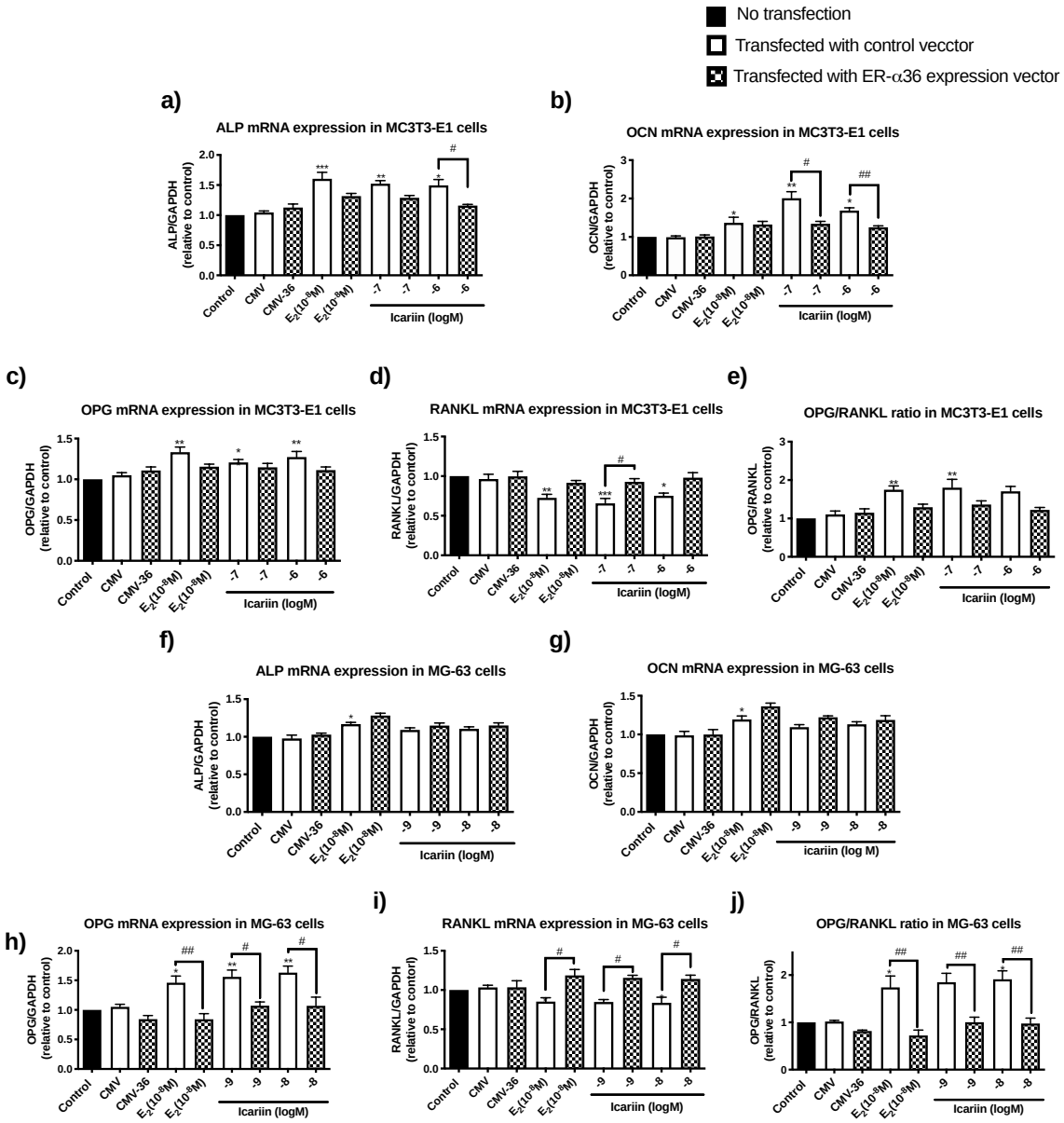


Figure 4.10 Effect of ER-α36 overexpression on mRNA expressions of bone markers induced by icariin in osteoblasts.

MC3T3-E1 (a-e) and MG-63 cells (f-j) were treated with vehicle, E_2, or icariin for 24 hours in phenol red-free DMEM containing 1% cs-FBS. mRNA was harvested by Trizol reagent. mRNA expression of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio were amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control.

4.3.3.3 **Effect of ER-α36 overexpression on estrogenic signaling in icariin treated osteoblasts**

Our results (Figure 4.11) demonstrated that ER-α36 overexpression significantly decreased the phosphorylation of Akt and ER-α (Ser167) induced by icariin in MC3T3-E1 cells (P <0.05 vs. treatment group transfected with CMV). The suppressive effects of ER-α36 overexpression on phosphorylation of ER-α at Ser118 and ERK induced by icariin in MC3T3-E1 cells did not reach statistical significance. Similarly, ER-α36 overexpression reversed icariin-induced phosphorylation of ERK and ER-α (Ser167) in MG-63 cells (P <0.05 vs. treatment group transfected with ER-α36). The suppressive effects of ER-α36 overexpression on phosphorylation of Akt and ER-α66 at Ser118 induced by icariin in MG-63 cells did not reach statistical significance.

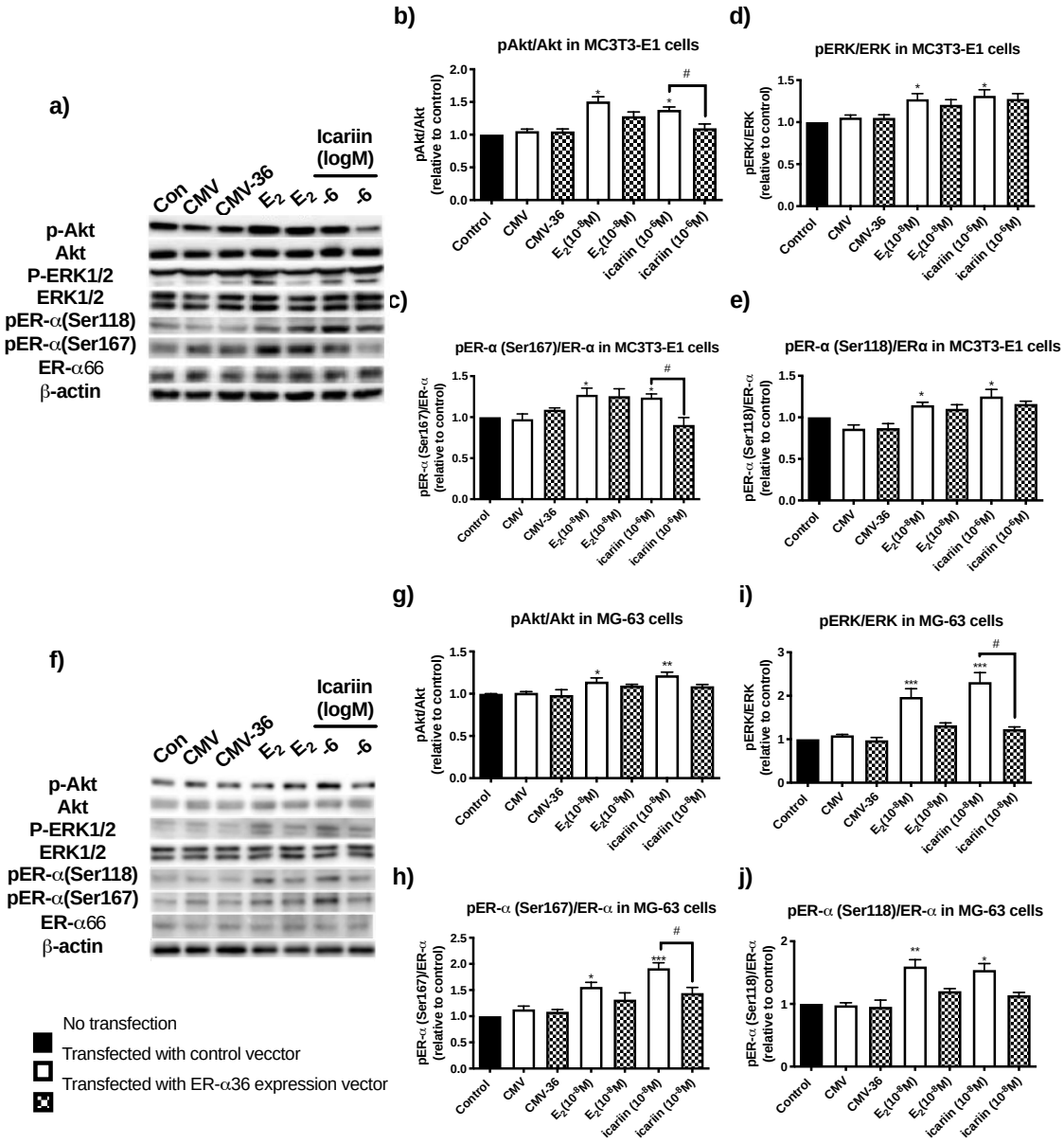


Figure 4.11 The effect of ER-α36 overexpression on rapid signaling induced by icariin in osteoblasts.

MC3T3-E1 (a-e) and MG-63 cells (f-j) were transfected with CMV or CMV-36 at desired dosages before treatment with vehicle, E_2, or icariin for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER-α (Ser167), pER-α66 (Ser118), and ER-α66 were studied by western blotting. The figures are the representatives of three independent experiments. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}$ $P < 0.05$ vs. treatment group transfected with CMV.

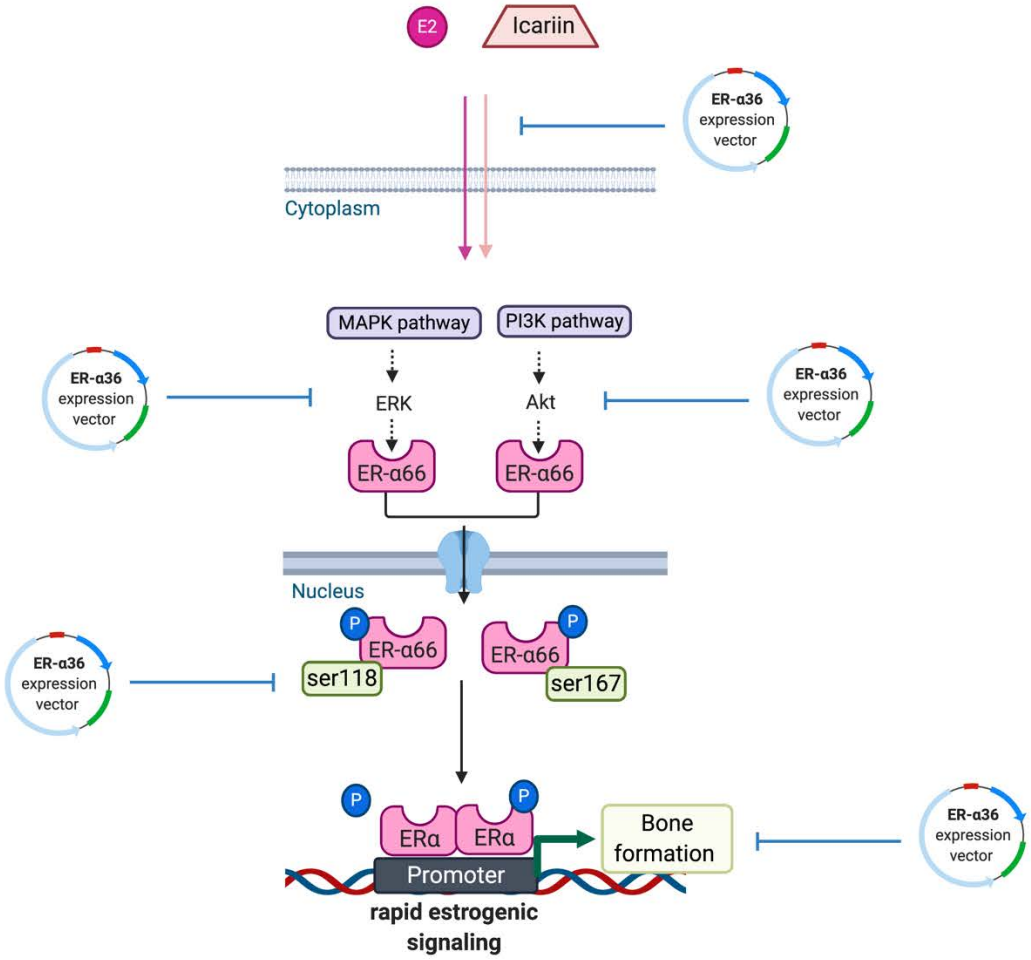


Figure 4.12 Summary of the role of ER-α36 in the actions of icariin in osteoblast. Overexpression of ER-α36 could reverse icariin-induced cell proliferation, mRNA expression of bone formation markers as well as activation of AKT and ERK pathway.

4.3.4 Characterization of the formation of signaling complexes amongst ERs upon treatment with icariin in osteoblastic cells

4.3.4.1 Recruitment of ERs upon treatment with E_2 and icariin in rBMSCs

Our results indicated that the fluorescence intensities of both GPER and ER-α36 were reduced in MC3T3-E1 cells upon icariin (10^{-7}M) and E_2 (10^{-8}M) treatment (Figure 4.13). At basal state, ER-α36 is located in the cytoplasm while GPER and ER-α66 are located in both the cytoplasm and the nucleus. E_2 and icariin treatment for 10 minutes triggered the translocation of ER-α66 from the cytoplasm to the nucleus; while ER-α36 and GPER seem to retain in the cytoplasm. Similarly, the fluorescence intensities of ER-α36 were remarkably reduced in rBMSCs upon treatment with E_2 (10^{-8}M) or icariin (10^{-7}M) for 10 minutes. E_2 likely induced the expression of GPER while icariin supressed it. At basal state, ER-α36 is located in the cytoplasm while GPER and ER-α66 were located in both the cytoplasm and the nucleus in rBMSCs. E_2 and icariin treatment triggered the translocation of ER-α66 from the cytoplasm to the nucleus in rBMSCs. Interestingly, both icariin and E_2 treatment triggered the translocation of ER-α36 and GPER from cytoplasm or nucleus toward the edge of the cell.

4.3.4.2 Effect of icariin on the formation of ER-dependent signaling complexes in osteoblasts differentiated from rBMSCs

Treatment of osteoblasts derived from rBMSCs with E_2 (10^{-8}M) and icariin (10^{-6}M) for 10 minutes increased the expression ER-α66, GPER, and shc (Figure 4.14). Immunoprecipitation assay successfully pulled out ER-α66 from total protein. Co-immunoprecipitation (Co-IP) assay suggested signaling complexes containing ER-α66, ER-α36, GPER, shc, and c-src were formed at the basal state of rBMSCs. Both E_2 and icariin treatment could reduce the formation of ER-α66/ER-α36 complexes but elevated ER-α66/shc formation in rBMSCs. Interestingly, only icariin treatment could suppress the formation of ER-α66/c-src in rBMSCs. No remarkable change in the amount of ER-α66/GPER in rBMSCs upon treatment with E_2 or icariin was observed.

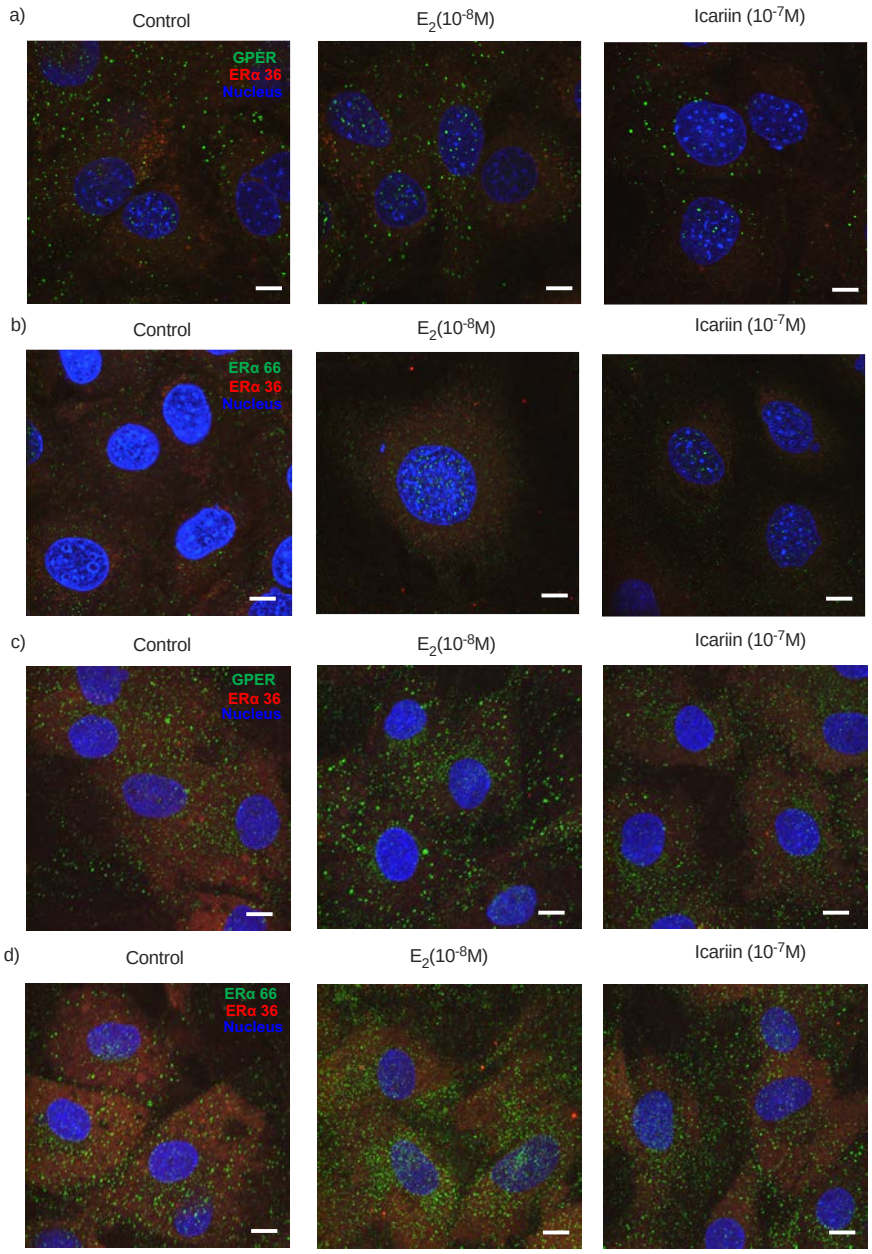


Figure 4.13 Effect of E_2 and icariin on the localization of ERs in osteoblasts.

MC3T3-E1 cells (a & b) and rBMSCs (c & d) were treated with vehicle, $E_2(10^{-8}M)$ or icariin ($10^{-7}M$) for 10 minutes. Treated cells were fixed immediately with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 1 min. Localization and translocation of ERs were determined by immunofluorescence staining. Double immunofluorescence staining and quantification of fluorescence intensities of ER-α36 and GPER or ER-α36 and ER-α66 in MC3T3E-1 cells (a-b) and rBMSCs (c-d) were shown. The scale bar represents 50μm.

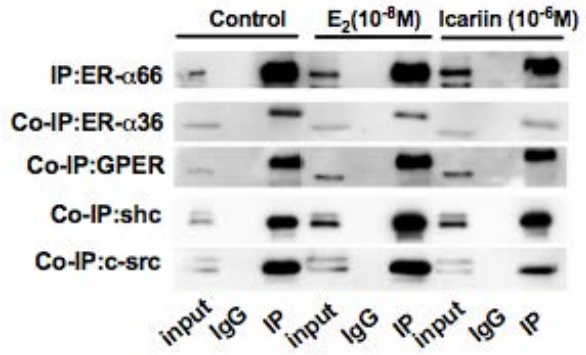


Figure 4.14 The effect of icariin on ER signaling complex formation in rBMSCs.

rBMSCs harvested from rat long bones were treated with vehicle, E_2, or icariin for 10 minutes. Total protein was extracted using lysis buffer. Signaling complexes among ER-α66, ER-α36, GPER, Shc, and c-src were studied using co-immunoprecipitation and western blotting. IgG was loaded as a negative control. The figures are the representatives of three independent experiments in rBMSCs.

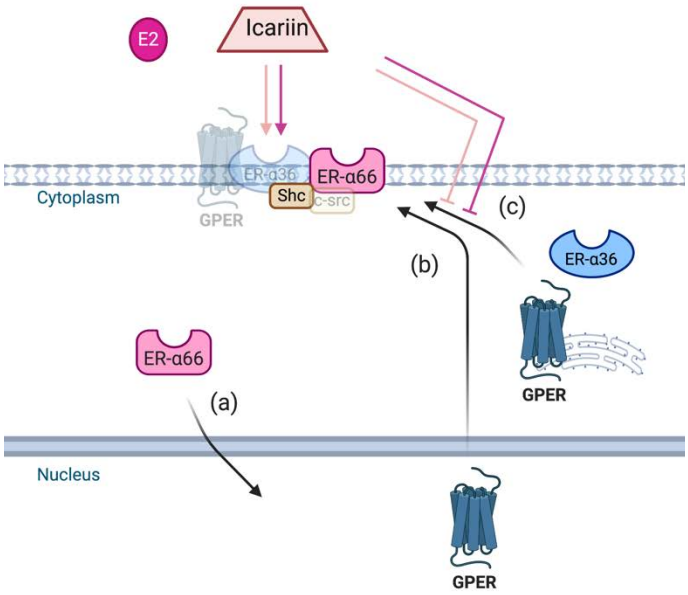


Figure 4.15 Summary of signaling transduction in osteoblast via ERs upon icariin treatment.

Icariin and E_2 could trigger the translocation of ER-α66 (a) from the cytoplasm to the nucleus while stimulate the translocation of GPER (b) and ER-α36 (c) to the cell membrane within 10 minutes of treatment. E_2 and icariin could suppress the formation of ER-α66/ER-α36 complex, while only icariin could reduce ER-α66/c-src in osteoblast.

4.4 Discussion

In this chapter, we have attempted to investigate the role of GPER and ER-α36 in the bone protective effects of icariin and E_2. We have shown that icariin works differently in recruiting GPER and ER-α36 in rapid estrogen signaling in osteoblasts (Figure 4.6). GPER and ER-α36 might work as negative collaborators to classical ER-α66 during bone formation.

GPER seems to be a negative regulator in bone remodelling that mediates the actions of icariin

Despite the role of GPER in bone remodelling has been briefly studied, no concrete conclusion could be drawn so far. In this study, we tried to study if GPER involve in mediating the effects of icariin on bone using MC3T3-E1 cells and MG-63 cells. They basally express a high level of ER-α66 and GPER. Blocking GPER with G15 and GPER knockdown appeared to enhance icariin-induced, but not E_2-induced, cell viability in both cells. Moreover, blocking GPER with G15 and GPER knockdown also promoted the mRNA expression of bone formation markers OPG induced by icariin, but not E_2, in both cells. Only the blocking of GPER with G15 could further enhance icariin-induced OCN in MG-63 cells, but not MC3T3-E1 cells. The differential effect of GPER on regulating OCN expression in these cells upon icariin might be due to different cell stages of these two cell lines. Pre-osteoblasts MC3T3-E1 cells mainly express ALP for osteoblast differentiation while MG-63 cells are mature osteoblasts that start to secret osteocalcin, an osteoblast-specific non-collagenous protein that binds calcium and hydroxyapatite during bone formation (Czekanska, Stoddart, Richards, & Hayes, 2012). Thus, the effect of GPER on OCN expression might be more sensitive in OCN-secreting mature osteoblast. The results indicated GPER involved in both E_2-indcued bone formation and icariin-induced bone formation and differentiation. We are the first to suggest that GPER seems to be a negative regulator in bone remodelling that mediates the actions of icariin and E_2 at molecular level.

ER-α36 is likely a negative regulator in ER-α66-mediated bone remodelling induced by icariin

On the other hand, ER-α36 overexpression not only significantly reduced icariin-induced expression of ALP and OCN mRNA in MC3T3-E1 cells, as well as the cell viability, the expression of OPG mRNA and OPG/RANKL ratio in MG-63 cells. RANKL mRNA expression induced by E_2 and icariin was significantly increased in ER-α36-overexpressed MG-63 cells. Overexpression of ER-α36 in MC3T3-E1 cells appeared to enhance bone differentiation induced by icariin while preventing the inhibitory effects of icariin on bone resorption. Taken together, ER-α36 seems to involve in mediating the actions of both E_2 and icariin in bone as a negative regulator. The negative involvement of ER-α36 in bone remodelling were found in a study by Wang et al. (Xie et al., 2011) in which knockdown of

ER-α36 further increased E_2-induced ALP and OCN mRNA expression in osteoblasts isolated from postmenopausal women.

ER-α36 might inhibit ER-α66-mediated bone protection of icariin and E_2. Previously shown by us and others (M. S. Wong & Zhang, 2013) that icariin could upregulate the cell viability and the expression of bone formation markers (such as OCN, ALP, OPG) in osteoblasts via ER-α66 activation. Results of the present study suggested that addition of ER-α36 could abolish these effects induced by icariin and E_2. These actions might anticipate the potential crosstalk among ERs. Formerly, ER-α36 was proposed to be a dominant-negative effector in estrogen-stimulated activation of estrogen-responsive genes through ER-α66 in MCF-7 cells (Y. Gu et al., 2014). ER-α36 contains a protein dimerization domain that allows it to form a heterodimer with ER-α66. ER-α66/ER-α36 heterodimer could restrain more ER-α66 in the cytoplasm, preventing it from activating nuclear transcription activities in MCF-7 cells (Z. Y. Wang & Yin, 2015). Thus, ER-α36 might form ER-α66/ER-α36 to inhibit ER-α66 action in bone. Interestingly, Wang's research proposed that low E_2 level (10^{-11}M), but not normal E_2 level (10^{-8}M), requires ER-α36 to promote cell proliferation and bone formation in ER-α66-low osteoblast derived from postmenopausal women (Xie et al., 2011). Our results showed that ER-α36 is a negative regulator in E_2-mediated bone protective effect in ER-α66-rich MC3T3-E1 cells and MG-63 cells. Therefore, the proposed estrogenic actions of ER-α36 by Wang et al. might only happen in bone when both E_2 level and ER-α66 expression is low. A follow-up study on the estrogenic effect mediated by ER-α36 upon icariin using ER-α66 KO mice will be discussed in chapter 6.

<u>GPER is likely a negative regulator in icariin's action on rapid estrogenic signaling</u>

Blocking GPER further increased the phosphorylation of Akt, ERK, and ER-α (Ser167) in MC3T3-E1 cells and MG-63 cells upon E_2 treatment for 10 minutes and boosted icariin-induced phosphorylation of ERK and ER-α (Ser167) in MC3T3-E1 cells. Similarly, GPER knockdown caused reinforcement of the phosphorylation of Akt in both cells upon treatment with icariin for 10 minutes. These results are in agreement with other studies that GPER has been classified as ER because it could be activated by estrogen and activate several estrogenic pathways, including the activation of ERK1/2 signaling in the nervous system (Prossnitz & Barton, 2011), ER-α66 pathway in the uterus, PI3K/Akt pathway in breast, and cAMP and calcium mobilization in breast and neuron (Gaudet et al., 2015). These studies provide evidence that GPER participates as an inhibitory role in mainly Akt and ERK pathway in osteoblastic cells in response to icariin. The suppression of P13K/Akt and ERK/MAPK pathways by GPER might interfere with the bone protective actions of icariin. It is widely studied that PI3K/Akt

and ERK/MAPK signaling pathway plays an essential role in regulating cell proliferation, ALP activity, calcium accumulation, and mRNA expression of OCN and OPG in bone remodelling (Xi et al., 2015). Therefore, icariin seems to downregulate GPER expression, thereby reducing its inhibitory effect on osteoblastogenesis via phosphorylation of Akt and ERK.

ER-α36 is likely a negative regulator in ER-α66-dependent signaling upon icariin

Overexpression of ER-α36 inhibited rapid estrogenic signaling, including the phosphorylation of Akt and ER-α (Ser167) in MC3T3-E1 cells and ERK and ER-α(Ser167) in MG-63 cells upon 10-minute treatment with icariin. In contrast, ER-α36 overexpression did not significantly alter the phosphorylation of measured signaling molecules upon E_2 treatment in both cells. These results suggest that ER-α36 negates the actions of icariin in bone and that the actions of icariin are different from E_2 in osteoblastic cells. The involvement of ER-α36 in MAPK/ERK pathways are reported by others in which ERK2 could directly bind to the domain D of ER-α36 that could in turn be recognized by its activators, substrate, and regulators for both phosphorylation and dephosphorylation. Such a structural finding suggests that ER-α36 could either activate or deactivate the MAPK/ERK pathway (Omarjee et al., 2017).

In particular, ER-α36 seems to inhibit ERK phosphorylation in cells with high ER-α66 expression. Our results showed that ER-α36 seems to be a suppressor in the ERK pathway, such suppression is opposite to the findings of others using ER-α66-low or ER-α66-negative cells. ER-α36 was suggested to be involved in tamoxifen-resistance via phosphorylation of ERK and Akt pathway in ER-α66-negative breast SK-BR-3 cells (Lin et al., 2010). Moreover, ER-α36 was also reported to mediate the phosphorylation of ERK in low ER-α66/ER-α36 ratio osteoblast isolated from Chinese postmenopausal women (Xie et al., 2011). The activation of MAPK/ERK pathway via ER-α36, in the absence of ER-α66, was also found in HEK293 cells treated with anti-estrogen, including tamoxifen and ICI 182,780 (Lianguo Kang et al., 2010). The knockdown of ER-α66 in endometrial cancer Hec1A cells did not alter ER-α36-mediated phosphorylation of MAPK/ERK induced by tamoxifen, resulting in the growth of endometrial cancer (Lin et al., 2009). It is of interest to note that MAPK/ERK pathway could be activated by icaritin, a metabolite of icariin via glycosylation, via ER-α36 in ER-α66-negative breast cancer cells, thereby inhibiting the growth of breast cancer (X. Wang et al., 2017). These conflicting reports regarding the role of ER-α36 on ERK activation may be due to the level of ER-α66 expression in different types of cells. Those cells showing activation of ERK and Akt by ER-α36 are low in ER-α66 (Y. Gu et al., 2014). Whereas, results from present study shown that ER-α36 placed a negative effect on ERK activation in high ER-α66/ER-α36-ratio MC3T3-E1 cells and MG-63 cells which suggest the phosphorylation of ERK by might depend on ER-

α66 expression. Based on differential regulation and crosstalk among ERs, ER-α36 is likely a negative regulators of ER-α66 in bone protection mediated by icariin and E_2. Further study on the ER-α36 role in ER-α66-negative osteoblasts will be illustrated in chapter 6.

Icariin stimulated the ERK or Akt pathway by inhibiting the formation of signaling complexes of ER-α66/ER-α36 and ER-α66/c-src.

We confirmed that ER-α36 expression was downregulated by icariin and E_2 in MC3T3-E1 cells and rBMSCs within 10 minutes of treatment. Also, ER-α36 and GPER were initially, at rest, located in the cytoplasm and moved toward the edge of the cell upon treatment with icariin and E_2 while ER-α66 was located both in the cytoplasm and the nucleus and translocated to the nucleus upon treatment with icariin in both cells. These results are compatible with others that ER-α66 could activate both rapid and genomic estrogen signaling in bone by the formation of ER dimers, followed by translocation to the nucleus in bone (Vrtačnik et al., 2014). Similar observations were also found in GPER-positive breast cancer cells in which ER-α36 mainly located on cell membranes for GPER-mediated rapid estrogen signaling (Lianguo Kang et al., 2010). Thus, icariin might activated ER-α66-mediated rapid estrogen signaling via the decrease in ER-α36 translocation to cell membrane and the increase in nuclear translocation of ER-α66. Apart from translocation, icariin could reduce the formation of ER-α66/ER-α36 and ER-α66/c-src complexes but increase ER-α66/shc formation in rBMSCs. Interestingly, only icariin could suppress the formation of ER-α66/c-src in rBMSCs upon treatment for 10 minutes while the amount of ER-α66/GPER was not changed. A previous study indicated that ER-α36 strongly inhibited the transcriptional activities of AF-1 and AF-2 domain in ER-α66 and retained ER-α66 in the cytoplasm to block its genomic signaling in the nucleus (Su et al., 2014). Therefore, it is possible that the reduction in ER-α36 expression and ER-α66/ER-α36 complexes by icariin enable ER-α66 to activate genomic estrogen signaling in rBMSCs. Furthermore, ER-α66/c-src complexes were diminished in rBMSCs upon icariin treatment. c-src, an upstream signaling molecule in MAPK/ERK or PI3K/AKT pathway (Franke, 2008), has been reported to be a negative regulator in osteoblastogenesis in which knockdown of c-src was previously shown to enhance osteoblastic cell proliferation, differentiation and bone formation *in vivo* (Marzia et al., 2000). Suppressing ER-α66/ER-α36 and ER-α66/c-src complexes in osteoblasts upon icariin treatment might prevent ER-α36 from interacting with c-src. In other words, the downregulation of ER-α66/c-src complex by icariin in rBMSCs might interrupt ER-α36 signaling transduction. Taken together, icariin could reduce ER-α66/ER-α36 and ER-α66/c-src complexes, allowing the translocation of ER-α66 to the nucleus for genomic and rapid estrogen signaling.

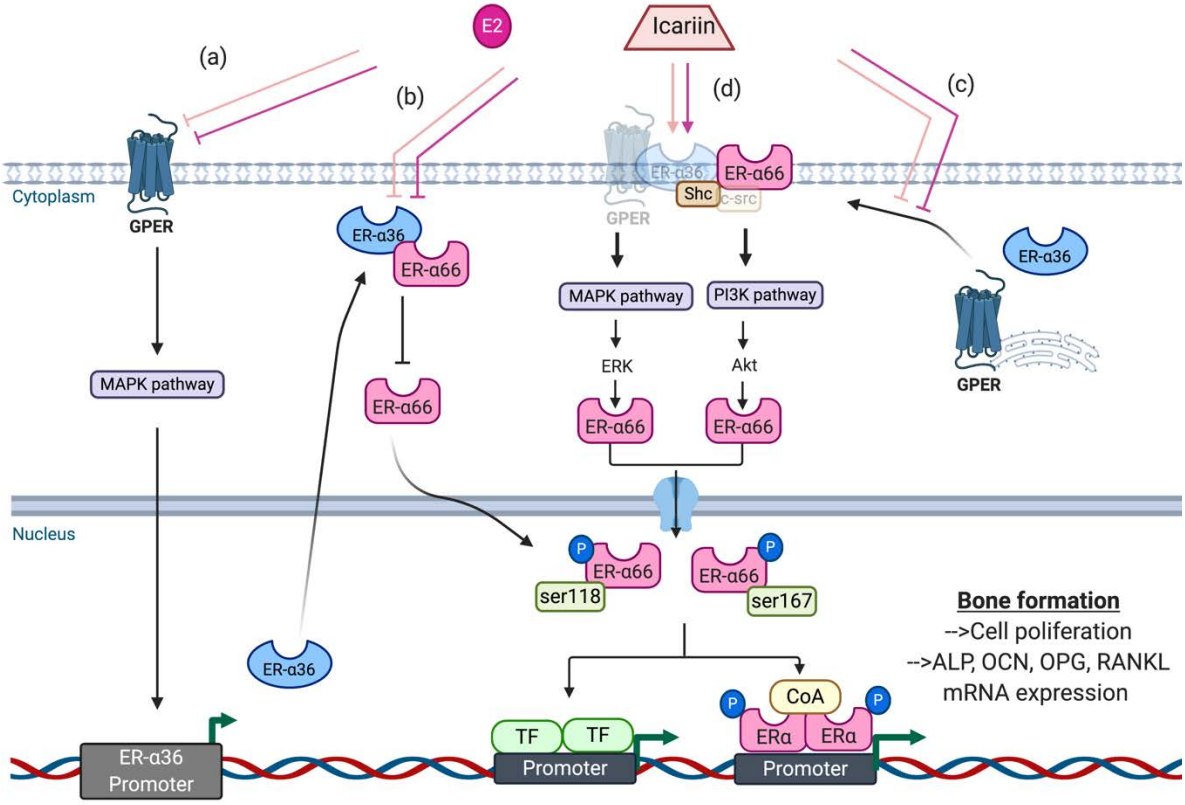


Figure 4.16 Schematic mechanisms illustrating the involvement of ERs in response to E_2 and icariin in bone.

E_2 and icariin stimulate osteoblastogenesis by activating the estrogen signaling pathway via complex interaction among ERs. First, E_2 and icariin supressed ER-α36 and GPER protein expression as well as activation of ER-α36 promoter activation by GPER (a). The decreases in ER-α36 expression leaded to less ER-α36/ ER-α66 heterodimer which might promote the translocation of free ER-α66 to nucleus for estrogen signaling (b). E_2 and icariin could also reduce the translocation of ER-α36 and GPER from cytoplasm to cell membrane (c) and remove ER-α36, GPER and c-src from ER-α66 signaling molecules which facilitated the activation of rapid estrogen signalling mediated by ER-α66 (d).These cooperative regulations of ERs by E_2 and icariin in osteoblast could ultimately promote bone formation.

Chapter 5. Characterization of the involvement of GPER and ER-α36 in bone protective effects of EXD

5.1 Introduction

Postmenopausal osteoporosis is by far the most common osteoporosis due to estrogen deficiency during menopause. Current treatment, including hormone replacement therapy (HRT) and selective estrogen receptor modulators (SERMs) targeting estrogen signaling, displays serious adverse effects, namely breast and endometrial cancer (M. Gambacciani & Vacca, 2004). Complementary and alternative medicine for managing postmenopausal osteoporosis is needed. Traditional Chinese Medicine (TCM) is a naturally occurring plant-derived remedy with a safety profile and becoming popular for treatment of hormone-related diseases around the world (Che, Wong, & Lam, 2016).

According to the theory of Chinese Medicine, "Kidney" plays an important role in governing bone (Fung & Linn, 2017). Er-xian decoction (EXD), known as a kidney tonifying TCM, was designed by Professor Zhang Bo-na at the Shanghai University of Traditional Chinese Medicine in China, in 1950s as a contemporary formula. EXD comprises six herbs, including *Herba epimedii* (HEP), *Rhizoma curculiginls* (XM), *Radix Morindae* (BJT), *Rhizoma Anmarrhenae* (ZM), *Cortex Phellodendri* (HB) and *Angelica Sinensis* (DG) in a compositional ratio of 9:9:9:6:6:9. It has been used for treatment of menopausal syndrome, osteoporosis and age-associated disease in the past 60 years (H. Y. Chen et al., 2008; L. L. Zhong et al., 2013). Preclinical and clinical studies demonstrated that EXD could increase serum estradiol levels (Gonzalez-Robayna et al., 2000; Sze et al., 2009) without causing adverse effects on reproductive and central nervous systems (J.-Y. Li et al., 2017). Our group also advocated that EXD could tissue selectively exert estrogen-like effects in bone and brain without stimulating the growth of uterus and breast (unpublished). Moreover, EXD could increase bone formation and differentiation in bone-forming osteoblasts while suppressing the proliferation and tartrate-resistant acid phosphatase activity of bone-breaking osteoclasts (Qin et al., 2008). EXD was found to promote the self-renewal and prevent adipogenesis in bone mesenchymal stem cells derived from ovariectomized (OVX) mice (S. Liu et al., 2016). These findings confirmed the estrogen-like effect of EXD in bone.

EXD might exert bone protection via the activation of estrogen signaling. Networking pharmacology and metabolomic studies revealed that the bone protective effects of EXD might occur through the regulation of lipid and energy metabolism, oxidation system, calcium, and steroidogenesis (S. Wang et al., 2015). Our group has shown that the EXD could regulate estrogen signaling in osteoblasts, promoting bone formation via both estrogen-responsive element (ERE)-dependent genomic and rapid estrogenic pathways, namely MAPK and Akt

pathways (K. C. Wong et al., 2014). These studies indicated that the mechanisms behind the actions of EXD in bone were complex. Moreover, due to the complexity and interaction of the active components in EXD, the estrogen signaling and effectors regulated by EXD in bone are far from clear. Mechanistic study about the effects of EXD on restoring bone condition, estrogen signaling, as well as estrogen receptors expression upon estrogen deficiency could allow us to understand how EXD might exert tissue-selective bone protective effects *in vivo.*

Novel membrane estrogen receptor alpha 36 (ER-α36) and G-coupled estrogen receptor modulator (GPER) might be involved in mediating the actions of EXD in bone. These two receptors have been reported to exert rapid estrogenic signaling in many estrogen-sensitive tissues (Gaudet et al., 2015; Soltysik & Czekaj, 2015). GPER is a seven-transmembrane-spanning estrogen receptor in which its estrogenic signaling activity and ligand binding occur upon intracellular trafficking from rough endometrium reticulum to the cell membrane (Gaudet et al., 2015). The activated GPER in turn triggers intracellular cAMP production and activation of PI3K/Akt and ERK1/2 pathways (Prossnitz & Barton, 2011). On the other hand, ER-α36, a novel isoform of classical estrogen receptor alpha 66 (ER-α66), has the ability to mediate rapid estrogenic pathways, namely ERK and Akt pathways, in breast or brain in response to estrogen (Soltysik & Czekaj, 2015). Moreover, the expression of GPER and ER-α36 was also reported to be associated with bone growth. Knockdown of GPER in mice was found to increase bone mass, femur size, cortical thickness, and mineralization (Ford et al., 2011). Whereas, the upregulation of ER-α36 was also found to be associated with the occurrence of postmenopausal osteoporosis in a clinical study (Xie et al., 2011). Thus, these studies suggest that extranuclear ER-α36 and GPER, besides ER-α66, could be possible candidates for mediating rapid estrogenic signaling induced by EXD on osteoblastogenesis.

Icariin is the most abundant phytoestrogen found in *Herba epimedium,* the principal drug in EXD and is believed to play an important role in governing the action of EXD (Y. Hu, Sze, Zhao, & Tong, 2009; Qin et al., 2008). With the evidence in Chapter 3 and 4, ER-α36 and GPER seem to work together to exert negative feedback on the actions of ER-α66 in osteoblastogenesis induced by icariin. It is of great importance to investigate if EXD recruits these ERs for signaling activation and bone formation in osteoblasts. Therefore, this chapter aimed at studying how ER-α36 and GPER respond to and mediate the bone protective effects of EXD (figure 5.1). It is hoped that this chapter could bring a better understanding of the estrogen-like effects of EXD on bone.

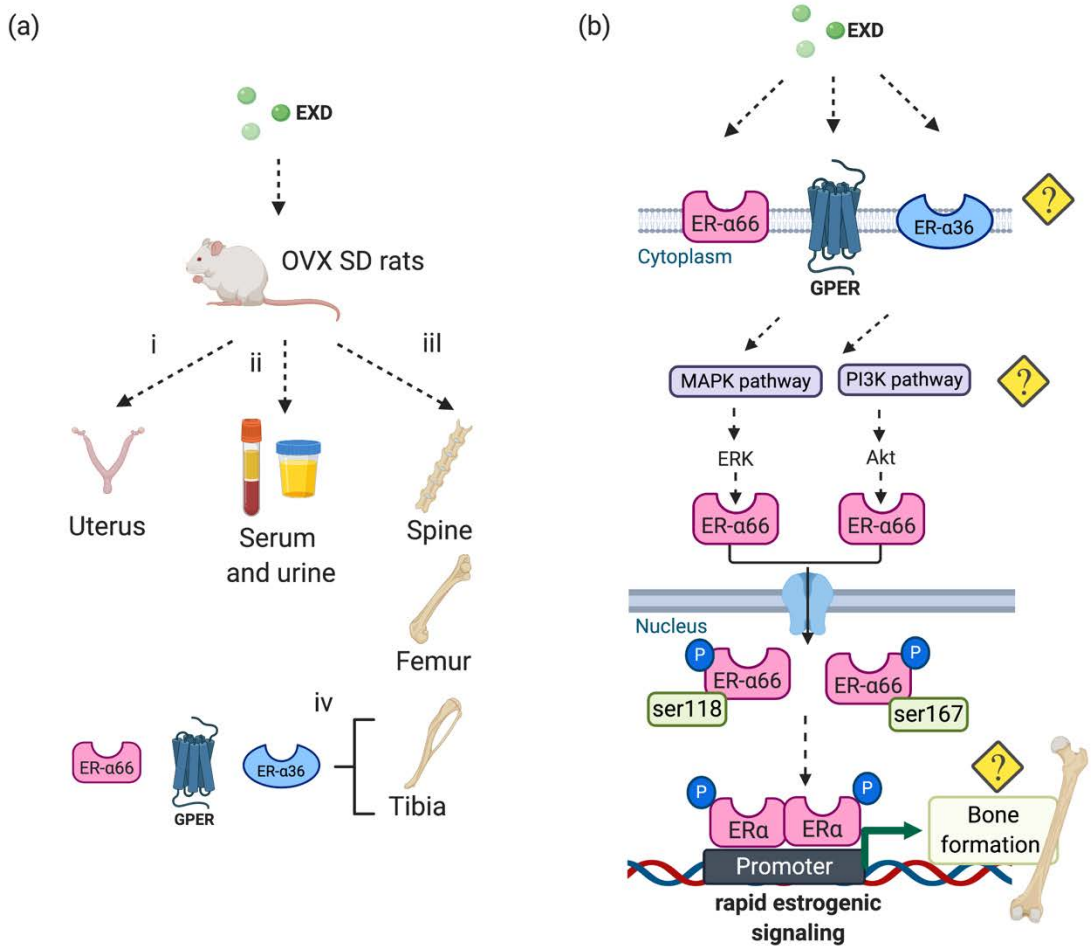


Figure 5.1 Overview of chapter 5.

In vivo (a), the effect of EXD in uterus (ai), serum hormone level, bone marker level as well as urine bone marker level (aii) and bone properties (aiii) at spine, distal femur and proximal tibia will be discussed. In particular, the protein expression of ERs in tibia heads of EXD-treated OVX rats will be illustrated (aiv). *In vitro* (b), The regulation of ER-α36, ER-α66 and GPER protein expression in EXD- or E_2-treated osteoblast *in vitro* will be discussed. The activation of estrogen signaling via these ERs by EXD as well as endpoint study of osteoblastogenesis mediated by these ERs will be explained.

5.2 Methodology

5.2.1 Authentication, extraction and quality control of EXD extract

EXD consists of six Chinese herbs, including *Herba epimedium* (Yin Yang Huo, HEP), *Rhizoma curculiginis* (Xianmao, XM), *Radix morindae officialis* (Bajitian, BJT), *Rhizoma anemarrhenae* (Zhimu, ZM), *Cortex phellodendri* (Huangbo, HB) and *Radix Angelicae Sinensis* (Danggui, DG) at the weight compositional ratio of 9:9:9:6:6:9. Six herbs were collected or purchased in the specific growing areas (Table 6.1) with the support of Professor Xinsheng Yao at Jinan University. High-performance liquid chromatography (HPLC) assays were conducted to ensure the qualities of herbs fulfil the requirement of the China Pharmacopoeia and/or the Hong Kong Chinese Materia Medica Standard. After authentication, herbs were delivered to Xi'an Pincredit Bio-tech Co., Ltd for extract preparation. The extracts were prepared according to the ancient recipe (K. C. Wong et al., 2014; J.-x. YANG, HUANG, CHEN, & WAN, 2013). Herbs were mixed at a given ratio and soaked in 8 volumes of water (v/w) for 2 hours, followed by boiling for 2 hours. These processes were repeated three times. The decoctions were then concentrated and spray-dried.

5.2.2 *In vivo* study on rats

5.2.2.1 Experimental designs

The animal experiment protocol was conducted under the animal license issued by the Department of Health, Hong Kong Special Administrative Region Government, and approved by the Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (animal license No. 18-168; ASESC Case: 15-16/31-ABCT-R-HMRF). Three-month-old female Sprague Dawley (SD) rats were purchased from the Chinese University of Hong Kong and raised for three months in Centralised Animal Facilities in the Hong Kong Polytechnic University. All rats were housed in a light-controlled (12h light and dark cycle) and temperature-controlled (22°C) environment. Six-month-old female SD rats were ovariectomized or sham-operated. After the operation, the rat diets were changed from laboratory autoclavable rodent 5010 (Labdiet, USA) to phytoestrogen-free AIN-93M (Research Diet, USA) to minimize the background of phytoestrogen in the diet. Rats were randomly divided into four groups after two-week recovery, and orally administered with the vehicle, 17-beta-estradiol (2mg/kg body weight/day) and EXD (1.6g/kg body weight/day). Weights of rats were measured every two weeks to monitor their health. Upon sacrifice, the uterus was collected and weighted to measure the uterus index. Serum was collected to investigate hormone modulation by EXD, while urine, right leg, and spine were collected to

study the bone protective effect of EXD in OVX rats. Left tibia heads were collected to measure the tissue responsiveness of ERs expression upon EXD treatment.

5.2.2.2 **Drugs preparation and animal feeding**

Upon a two-week recovery from ovariectomy, rats were randomly arranged into four groups (10 rats/group) for 12-week treatment and paired-fed with phytoestrogen-free AIN 93 diet. Sham-operated rats and one group of OVX rats were given water as a control group and negative control group, respectively. One OVX group was orally administered with 17ß-estradiol (E_2, 2mg/kg body weight/ day, Sigma, Cat#E8875) dissolved in distilled water in forms of suspension as a positive control. Another OVX group was orally administered with EXD (1.6g/kg body weight/ day) dissolved in distilled water. These drug dosages were chosen based on the effective doses used in our published study(K. C. Wong et al., 2014).

5.2.2.3 **Sample collection**

Weights of rats were measured every two weeks after surgery. Twenty-four hours before sacrifice, rats were individually housed in metabolic cages for urine collection. Urine was centrifuged at 4000rpm/min for 15 minutes, and the supernatant was stored at -80°C for further measurement. At sacrifice, blood was drawn from the abdominal aorta under anaesthesia with 50mg/kg ketamine (Alfasan, The Netherlands) and 10mg/kg xylazine (Alfasan, The Netherlands), and aliquots of serum were collected after centrifugation at 4000rpm at 4°C for 15 minutes. The serum was then stored at -80°C for measuring the hormone level. Uterus was freshly removed and weighed for assessing the uterus index. Right legs and spines were wrapped with gauze soaked with PBS and stored at -80°C for micro-CT. Left tibias and femurs were collected and stored in liquid nitrogen for western blotting.

5.2.2.4 **Haematoxylin-Eosin (H&E) staining**

The uterus was removed from sham and OVX rats treated with vehicle, E_2, or EXD and fixed with 4% paraformaldehyde. After tissue processing (TP1020 tissue processor, Leica, United States), tissues were embedded in paraffin (Surgipath Paraplast, Leica, United States, Cat#39601006). 5um-thick tissue sections were produced for each sample using a microtome (HistoCore BIOCUT Manual Rotary Microtome, Leica, United States). To observe the structural changes within the uterus in response to treatments, H&E staining was performed according to the manufacturer's instructions. A minimum of 5 sections from each sample was observed at 100X magnification and photographed using a digital microscope camera (DMC4500, Leica, United States).

Table 5.1 Collection of herbs in Er-Xian decoction.

Herbs	Partition	Growing area	Ratio
Herba epimedium	Whole herb of Epimedium brevicornum Maxim	Sichuan Wangcang county	9
Rhizoma curculiginis	Rhizome of Curculigo orchioides Gaertn	Anhui Qingyuan City	9
Radix morindae officialis	The root of Morinda officinalis How	Guangdong Qingyan City	9
Rhizoma anemarrhenae	Rhizome of Anemarrhena asphodeloides Bge	Hebei Anguo City	6
Cortex Phellodendri	Bark of Phellodendron chinese Scheneid	Sixhuan Emei City	6
Radix Angelicae sinensis	Root of Angelica sinensis (Oliv.) Diels	Gansu Minxian	9

5.2.2.5 **Bone mineral density (BMD) and Micro-CT analysis**

Bone properties of trabecular bone at proximal tibia and distal femur, as well as a lumbar vertebra (L4), were determined in the present study. The bones at three sites were scanned in the axial direction with a medium resolution of 21 m and a scanning power of 70 kVp and 110A. A total number of 200 slices per scan was done. The scanning positions of distal/proximal long bone were defined as 4.2mm and 2.2mm away from femur/tibia head. From the slide where the growth plates disappear, 100 consecutive slices away from the growth plate were analyzed. Lumbar vertebra (L4) was scanned in the middle of the bone for 250 slices (middle point ± 125 slices). The middle 150 slices were used for analysis. The threshold for contouring volume of interest of trabecular bones is based on the adaptive method, in which the contoured images were matched with the grayscale of the background image. The threshold values for contouring trabecular bones were 375. Evaluation Program v6.0 (Scanco Medical, Switzerland) generated bone biological parameters, including bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular bone number (Tb.N, 1/mm), trabecular bone thickness (Tb.Th, mm), trabecular bone separation (Tb.Sp, mm), connectivity density (Conn.D, $1/mm^3$) and structure model index (SMI). Morphological figures of the processed volume of interest (VOI) images were generated for a three-dimension figure presentation.

5.2.2.6 **Western blotting**

Protein expression of ER-α66, ER-α36, and GPER were studied. Details were described in Chapter 3.

5.2.2.7 **Serum hormonal levels and urinary bone markers**

Serum level of E_2, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), osteocalcin (OCN) were determined by Estradiol EIA Kit (CayMan, United States, Cat#582251), ELISA kits for FSH (Cloud Clone Corp, Cat#CEA830Ra) and LH (Cloud Clone Corp, United States, Cat#CEA441Ra), ELISA kits for OCN (Alfa Aesar, Car#J65214), respectively. As for urinary bone marker, a biomarker for bone breakdown, deoxypyridinoline (DPD), in urine was measured by DPD EIA kit (QUIDEL Corporation, United States, Cat# 8007). The urinary level of DPD was normalized by creatinine that was determined by picric acid methods using commercial kits (Zhongsheng Beikong Bio-technology and Science Inc., Beijing, China).

5.2.3 *In vitro* study

5.2.3.1 Experimental design

Mouse pre-osteoblast MC3T3-E1 cells and human osteoblast-like MG-63 cells were used to investigate the role of ER-α36 and GPER in the action of EXD in the pre-osteoblastic and mature osteoblastic stage, respectively. First, mRNA and protein expression of ER-α36, ER-α66, GPER were examined using real-time PCR and western blotting in response to E_2 at 10^{-8}M, EXD (0.1 to 100ug/ml). Besides, overexpression of ER-α36, GPER knockdown, or pre-treatment of G15, a GPER specific antagonist, was performed in these cells to assess the involvement of ER-α66, GPER in the bone protections of EXD. MTS assay, mRNA expressions of bone markers, and phosphorylation of signaling molecules in Akt and MAPK pathways were studied.

5.2.3.2 MTS Assay

MC3T3-E1 cells and MG-63 cells were as cultured as described in chapter 3. Cells were seeded in 96-well plates and cultured for 24 hours. Different cell models (ER-α36 overexpressed cells, GPER knockdown cells, as well as G15 pre-treatment cells) were established, as mentioned before. EXD at various concentrations (0.1 to 100ug/ml), E_2 at 10^{-8}M or its vehicle were added to the cells for 24 hours, followed by the determination of cell proliferation using MTS assay as described in chapter 4.

5.2.3.3 Real-time PCR

mRNA expression of estrogen receptors in MC3T3-E1 cells and MG-63 cells upon 24-hour treatment of vehicle, E_2 at 10^{-8}M, EXD at 0.1 to 100ug/ml were determined by real-time PCR with primers listed in chapter 3. Moreover, mRNA expression of different bone markers was studied, including osteoprotegerin (OPG) and osteocalcin (OCN) for bone formation, nuclear factor kappa-B ligand(RANKL) for bone resorption and alkaline phosphatase(ALP) for bone differentiation in different cell models treated with E_2 at 10^{-8}M and EXD at optimal doses using specific primers listed in table 5 in chapter 4. Details of mRNA extraction, reverse transcription, as well as real-time PCR, have been described in chapter 3.

5.2.3.4 Western blotting

MC3T3-E1 cells and MG-63 were treated E_2 at 10^{-8}M, EXD at optimal dose for 10 minutes. Protein expression of signaling molecules, including p-Akt, p-ERK, p-ER-α(Ser167), and (Ser118), were studied. Protein extraction and quantification, as well as western blotting, were performed as described in chapter 4.

with enhanced chemiluminescence reagent and visualized by Azure c600 (Azure Biosystems, USA).

5.2.3.5 **Statistical analysis**

Inter-group difference in *in vitro* study were determined by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. All graphs in this study were plotted by using GraphPad Prism Version 7.0.

5.3 Result

5.3.1 Authentication, extraction and quality control of EXD extract

Herbal extracts were authenticated as described based on the presence of specific chemical markers. They are Timosaponin BII for ZM, *Curculigoside* for XM, icariin for YYH, Berberine hydrochloride and Phellodendrine Chloride for HB, ferulic acid for DG, nystose for BJT and naringin for GSB. HPLC profile in Appendix 1 and quantities of standard in particular herb (Appendix 2) confirmed that the quality of all raw herbs have fulfilled the respective requirements in China Pharmacopeia in 2017. The amount of each chemical marker in EXD water extract was quantified by LC-MS (Appendix 3).

5.3.2 Characterization of the bone protective effect of EXD in OVX rats

5.3.2.1 Effect of EXD on body weight, hormone regulation and uterine growth

In figure 5.2, body weight of OVX rats appeared to increase without reaching statistical significance when compared to sham group. 12-week administration of E2, but not EXD, significantly suppressed OVX induction of body weight in rats (P<0.001 vs. OVX). As concerns hormone regulation, OVX surgery remarkably reduced serum E_2 level (P<0.05 vs. OVX) and boosted serum FSH (P<0.001 vs. OVX) and LH level (P<0.01 vs. OVX) in OVX rats. Administration of E_2 dramatically increased serum E_2 (P<0.05 vs. OVX) while suppressed serum FSH (P<0.01 vs. OVX) and LH level (P<0.001 vs. OVX) in OVX rats. EXD, acted like E_2, promisingly reversed OVX-induced changes in these serum hormone levels in rats (P<0.05 vs. OVX).

Uterus index dramatically decreased in rats upon ovary removal for 14 weeks (P<0.001 vs. OVX). Administration of E_2, but not EXD, promoted uterus weight of OVX rats. H&E staining confirmed that OVX surgery led to shrinkage of uterus lumen and formed a one-cell-thick uterine epithelial layer in rats when compared to the sham group. Administration of E_2 promoted the growth of epithelial cells and increased the thickness of the epithelial layer in OVX rats. Unlike E_2, EXD treatment did not alter the development of the uterine epithelial layer in OVX rats.

5.3.2.2 Effect of EXD on bone turnover biomarker in OVX rats

Ovariectomy significantly increased serum osteocalcin level by two-fold (P<0.001 vs. OVX) and urinary deoxypyridinoline/creatinine level by three-fold (P<0.001 vs. OVX) in OVX rats (Figure 5.3). On the contrary, 12-week treatment with E_2 significantly suppressed the OVX-induced increase in both bone turnover biomarkers in OVX rats (P<0.001 vs. OVX) while EXD treatment could only restore serum osteocalcin level in OVX rats (P<0.001 vs. OVX).

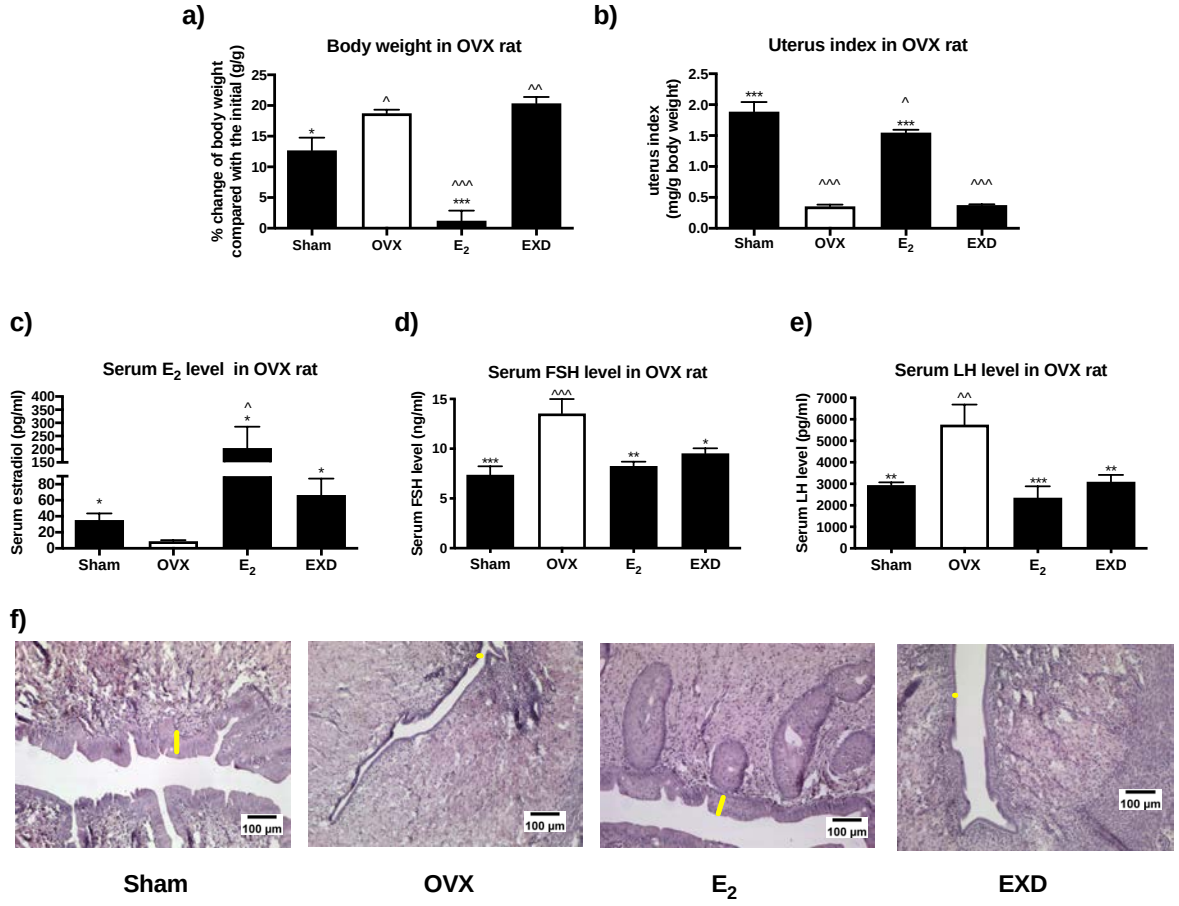


Figure 5.2 Effect of EXD on the uterus, body weight, and reproductive hormones in OVX Sprague Dawley (SD) rats.

Sham-operated and OVX SD rats were pair-fed with phytoestrogen-free AIN-93M diet and treated with vehicle, 17ß-estradiol (2mg/kg body weight/day) or EXD (1.6g/kg body weight/body) for 12 weeks. Changes in body weight (a) were recorded. Uterus was collected to examine the uterus index (b) by dividing the uterus weight by body weight of rats. Serum was collected to assess serum E_2 (c), FSH (d), and LH (e) level of the rat using ELISA. n=8-10. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. OVX; ^ $P < 0.05$, ^^ $P < 0.01$ and ^^^ $P < 0.001$ vs. Sham. Estrogenic effects on the uterus of rats have been studied using H&E staining (f). Pictures were captured using Leica DMC4500 at 100X magnification. Yellow lines indicated the thickness of the uterine epithelial layer.

5.3.2.3 **Effect of EXD on bone mineral density (BMD) and bone microarchitecture in OVX rats**

The effects of OVX, E_2, and EXD on bone mineral density (BMD) and microarchitectural properties at the proximal tibia, distal femur and lumbar vertebra (L4) of OVX rats were illustrated with their representative three-dimensional images (Figure 5.4) and parameters obtained from micro-CT analysis (Table 6.2). OVX significantly induced bone loss in the proximal tibia and distal femur metaphysis as well as in spine as indicated by the significant increase in structure model index (SMI), trabecular separation (Tb.Sp, mm) and decrease in bone mineral density (BMD, mg HA/ cm3), bone volume/tissue volume (BV/TV), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm) and connectivity density (Conn.D, 1/mm3) in rats ($P< 0.001$ vs. Sham). The OVX-induced bone deterioration in rats was prevented by treatment with E_2 and EXD. E_2 significantly reversed OVX-induced changes in all bone parameters ($P<0.05$ vs. OVX) while EXD improved OVX-induced bone loss by restoring BMD, BV/TV, Conn.D., SMI, Tb.N, and Tb.Sp at the lumbar spine (L4), BMD, Tb.N, and Tb.Sp at distal femur as well as BMD, Conn.D., Tb.Th at proximal tibia of OVX mice ($P<0.05$ vs. OVX).

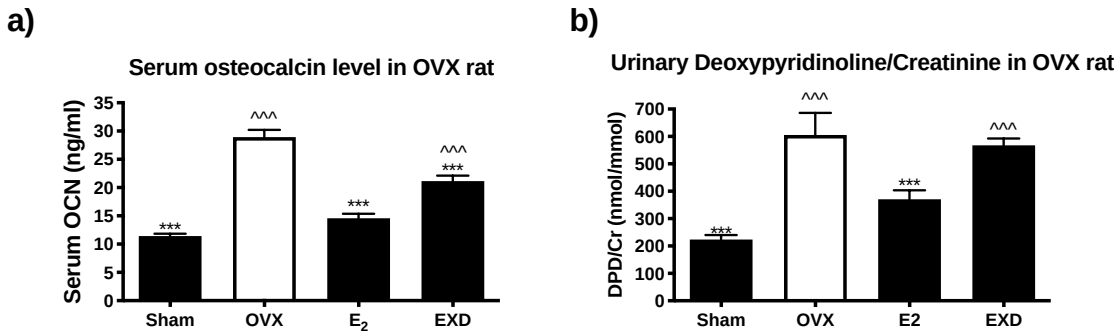


Figure 5.3 Effect of EXD on bone turnover biomarkers in OVX Sprague Dawley (SD) rats.

Sham-operated or and OVX SD rats were pair-fed with phytoestrogen-free AIN-93M diet and treated with vehicle, 17ß-estradiol (2mg/kg body weight/day) or EXD (1.6g/kg body weight/body) for 12 weeks. Serum osteocalcin level (a) and urinary Deoxypyridinoline/Creatinine level (b) in OVX rats were measured using ELISA. n=8-10. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. *** $P < 0.001$ vs. OVX; ^^^ $P < 0.001$ vs. Sham.

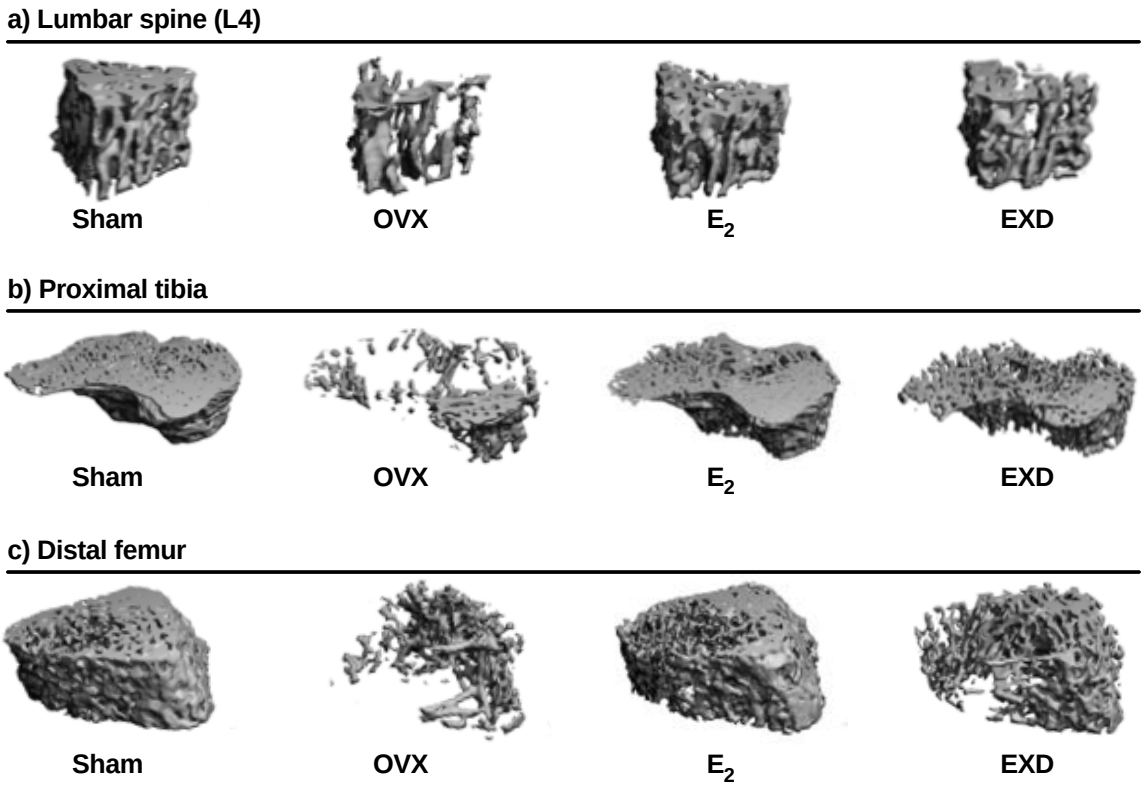


Figure 5.4 Effect of EXD on the bone microarchitecture of lumbar vertebra L4 (A), proximal tibia (B), and distal femur (C) in OVX Sprague Dawley (SD) rats.

Sham-operated or and OVX SD rats were pair-fed with phytoestrogen-free AIN-93M diet and treated with vehicle, 17ß-estradiol (2mg/kg body weight/day) or EXD (1.6g/kg body weight/body) for 12 weeks. Left tibia, femur, and lumbar vertebra were collected upon the sacrifice and scanned at high resolution by the micro-CT system (viva-CT40, Scanco Medical, Switzerland). A constant threshold of 375 was used to generate three-dimensional images for all samples.

Table 5.2 Effect of EXD on bone microarchitecture parameters of lumbar vertebra L4, proximal tibia, and distal femur in OVX Sprague Dawley (SD) rats.

Bone	Group	BMD (mg HA/cm3)	BV/TV (%)	Conn. D. (mm3)	SMI	Tb.N (mm-1)	Tb.Th (μm)	Tb.Sp (μm)
Lumbar spine (L4)	Sham	361.7±8.0	0.41±0.02	33.97±2.7	0.13±0.1	3.36±0.1	0.17±0.008	0.24±0.01
	OVX	183.4±16.1^^^	0.16±0.02^^^	12.62±2.6^^^	2.06±0.1^^^	2.04±0.1^^^	0.11±0.003^^^	0.47±0.03^^^
	E2	296.5±10.3***	0.34±0.05***	25.79±2.3***	0.57±0.3***	2.85±0.1***	0.14±0.007*	0.32±0.02***
	EXD	192.0±8.8*	0.27±0.02*	20.11±1.3**	0.85±0.2**	2.56±0.1**	0.12±0.003	0.37±0.01***
Proximal tibia	Sham	460.3±12.7	0.62±0.02	56.22±2.2	-2.04±0.4	4.69±0.1	0.17±0.008	0.15±0.06
	OVX	103.5±7.0^^^	0.11±0.02^^^	8.12±1.5^^^	2.56±0.1^^^	1.29±0.1^^^	0.11±0.003^^^	0.82±0.06^^^
	E2	363.5±19.3***	0.43±0.03***	46.87±1.9***	0.12±0.4***	3.73±0.1***	0.14±0.007**	0.23±0.02***
	EXD	192.0±8.8*	0.16±0.01	18.67±2.4	2.14±0.1	2.19±0.1**	0.12±0.003	0.46±0.02***
Distal Femur	Sham	460.3±12.7	0.51±0.02	44.61±2.7	-0.82±0.25	4.13±0.1	0.16±0.005	0.21±0.01
	OVX	103.5±7.0^^^	0.08±0.01^^^	11.69±2.05^^^	2.15±0.1^^^	1.56±0.1^^^	0.11±0.004^^^	0.66±0.02^^^
	E2	363.5±19.3***	0.39±0.04***	35.75±2.4***	-0.31±0.4***	3.20±0.2***	0.15±0.010***	0.29±0.03***
	EXD	209.4±10.2**	0.22±0.01***	21.60±2.0*	1.24±0.1	1.88±0.1	0.13±0.003*	0.54±0.02

Sham-operated or and OVX SD rats were pair-fed with phytoestrogen-free AIN-93M diet and treated with vehicle, 17ß-estradiol (2mg/kg body weight/day) or EXD (1.6g/kg body weight/body) for 12 weeks. Bone microarchitecture parameters include bone volume/tissue volume (BV/TV), connectivity density (Conn.D), structural model index (SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). Data were presented by mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. ^ $P < 0.05$, ^^ $P < 0.01$, and ^^^ $P < 0.001$ vs. Sham; and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. OVX.

5.3.3 **Effects of EXD on protein expression of different ERs in bone**

5.3.3.1 **Effect of EXD on different ERs protein expression in tibia heads of OVX rats**

Protein expression of ER-α66, ER-α36, and GPER was significantly increased in tibia head in adult female rats (P<0.001 vs. sham) (Figure 5.5). Upon treatment with E_2 or EXD for 12 weeks, the protein expressions of ER-α66, ER-α36, and GPER in tibia head were significantly reduced in OVX rats (P<0.001 vs. OVX).

5.3.3.2 **Effect of EXD on different ERs protein expressions in bone cells**

In figure 5.6, EXD mimicked the effect of E_2 to increase the protein expression of ER-α66 at 10μg/ml and 100μg/ml (P<0.05 vs. Control). Moreover, ER-α36 protein expression were significantly suppressed by EXD from the dose at 10ug/ml to 100ug/ml while GPER protein expression were significantly suppressed by EXD from the dose at 0.1ug/ml to 25ug/ml. (P<0.05 vs. Control). Similarly, E_2 treatment significantly increased the protein expression of ER-α66 by 1.2-fold (P<0.5 vs. Control) while EXD promoted its expression from 25μg/ml to 100μg/ml (P<0.5 vs. Control) in MG-63 cells. In contrast, E_2 treatment significantly suppressed the protein expression of both ER-α36 and GPER by 0.75-fold (P<0.05 vs. Control), while EXD dose-dependently reduced the protein expressions of ER-α36 and GPER by around 0.8-fold from 0.1μg/ml to 25μg/ml in MG-63 cells (P<0.05 vs. Control).

5.3.3.3 **Effect of EXD on different ERs mRNA expression in bone cells**

Due to the lack of information about the mRNA sequence of mice ER-α36, only GPER and ER-α66 mRNA were measured in MC3T3-E1 cells. Our results (Figure 5.7) demonstrated that E_2 at 10^{-8}M and EXD at 50μg/ml significantly increased mRNA expression of ER-α66 in MC3T3-E1 cells upon treatment for 24 hours (P <0.05 vs. Control). In addition, E_2 at 10^{-8}M tended to increase the mRNA expression of GPER, while EXD treatment tended to suppress it without statistical significance in MC3T3-E1 cells. On the other hand, E_2 at 10^{-8}M and EXD at 0.1 to 1μg/ml and 50 to 100μg/ml significantly stimulated the mRNA expression of ER-α66 (P<0.05 vs. Control), while E_2 at 10^{-8}M, but not EXD, remarkably suppressed ER-α36 mRNA expression in MG-63 cells (P<0.5 vs. Control). As for GPER, E_2 at 10^{-8}M significantly increased its mRNA expression (P<0.05 vs. Control), while EXD at high dose (50 or 100μg/ml) tended to reduce it but the changes did not reach statistical significance in MC3T3-E1 cells. Moreover, no significant modulation of GPER mRNA expression were observed in MG-63 cells upon E_2 at 10^{-8}M or EXD treatment.

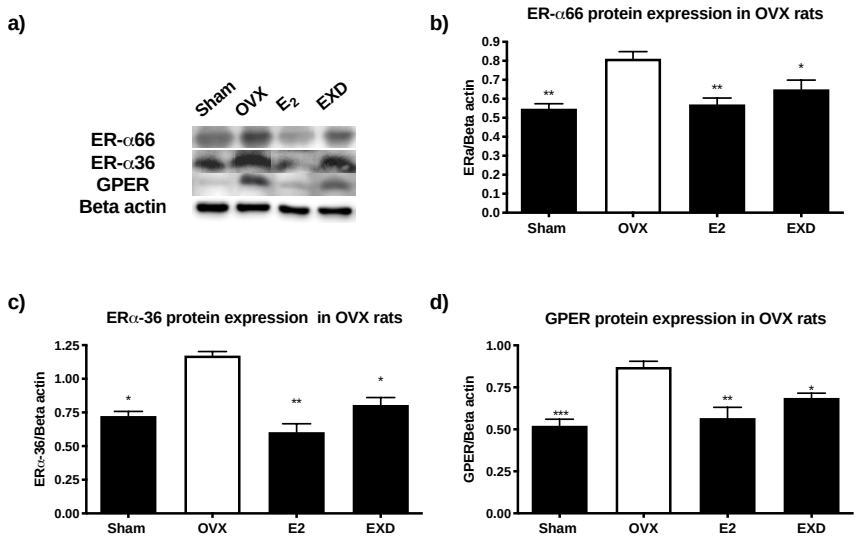


Figure 5.5 Effect of E_2 and EXD on ERs protein expressions in OVX Sprague Dawley (SD) rats.

Sham-operated or and OVX SD rats were pair-fed with phytoestrogen-free AIN-93M diet and treated with vehicle, 17ß-estradiol (2mg/kg body weight/day) or EXD (1.6g/kg body weight/body) for 12 weeks. Tibia heads were isolated from rats upon the sacrifice, and proteins were extracted to analyze the expression of ER-α66 (b), ER-α36 (c), and GPER (d) by western blotting. (a) Figures are the representative of three times of independent trials. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$ and ** $P < 0.01$vs. OVX.

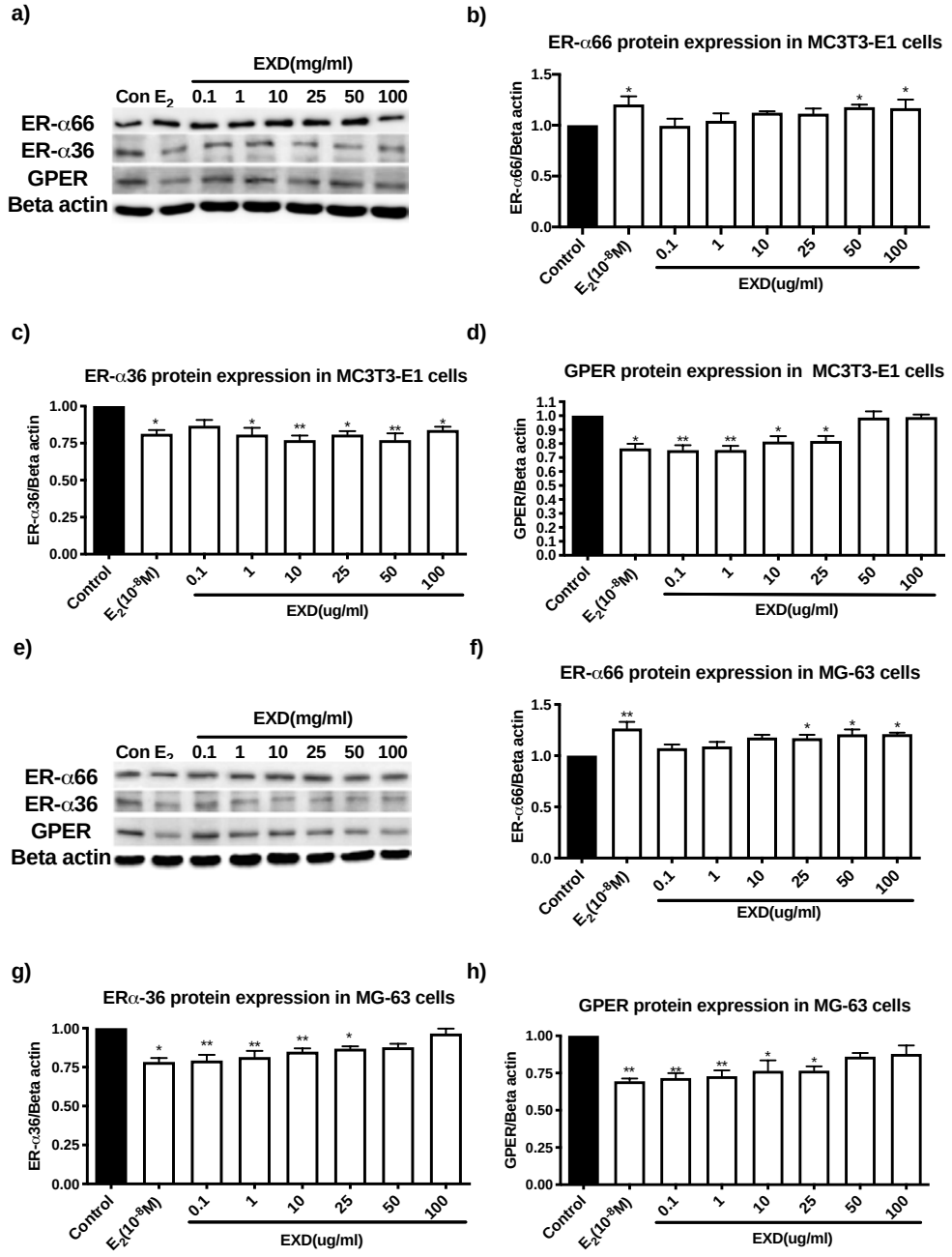


Figure 5.6 Effect of E_2 and EXD on ERs protein expressions in osteoblasts.

Murine MC3T3-E1 and human MG-63 cells were treated with vehicle, E_2 (10^{-8}M), or EXD (0.1 to 100μg/ml) for 24 hours in phenol red-free DMEM containing 1% cs-FBS. Proteins were extracted to analyze the expression of ER-α66, ER-α36, and GPER in MC3T3-E1 cells (b-d) and MG-63 cells (f-h). The figures (a and e) are the representatives of immunoblot from three independent trials in MC3T3-E1 cells and MG-63 cells, respectively. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ±

SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. OVX.

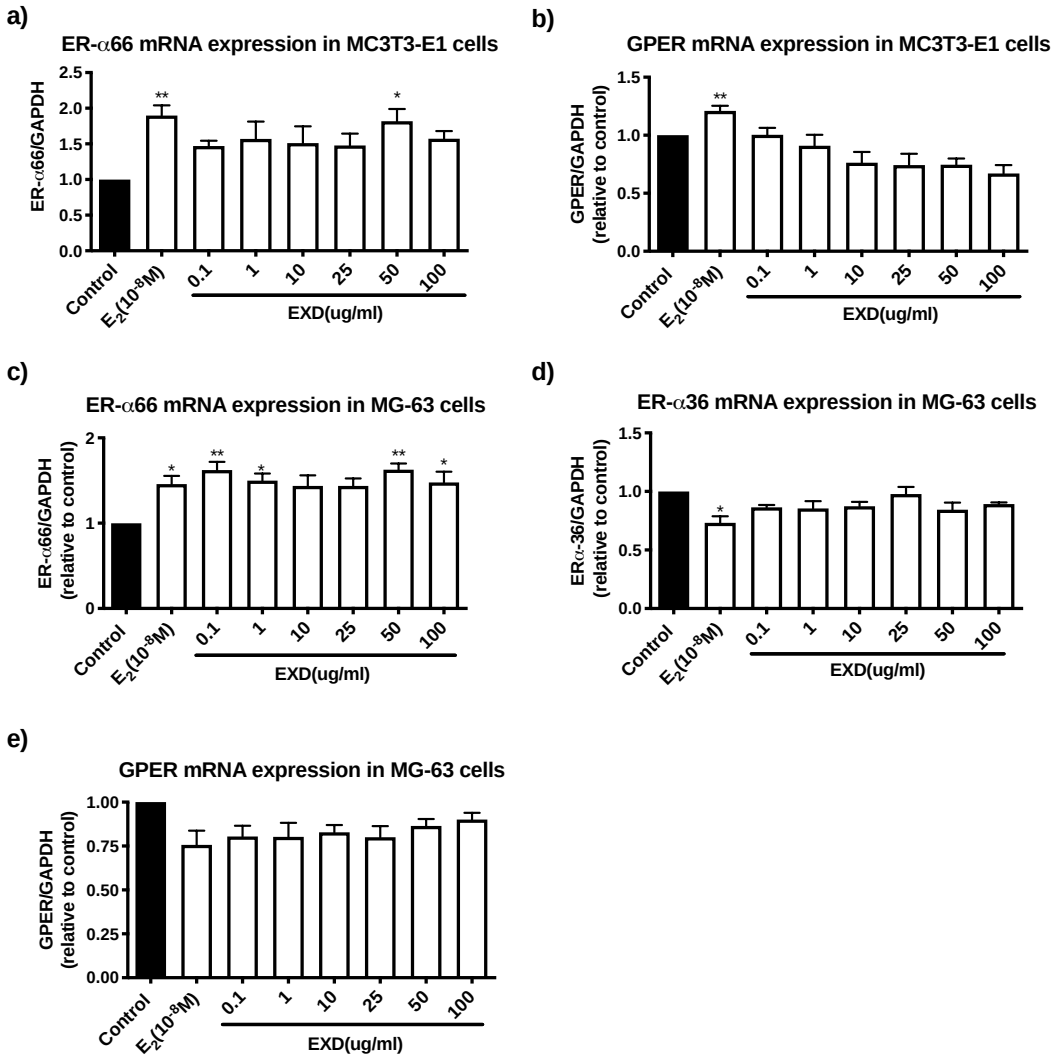


Figure 5.7 Effect of E_2 and EXD on ERs mRNA expressions in osteoblasts.

Murine MC3T3-E1 cells and human MG-63 cells were treated with vehicle, E_2 (10^{-8}M), or EXD (0.1 to 100μg/ml) for 24 hours in phenol red-free DMEM containing 1% cs-FBS. mRNA was harvested by Trizol reagent. mRNA expression of ER-α66 and GPER in MC3T3-E1 cells (a-b) and that of ER-α66, ER-α36, and GPER in MG-63 cells (c-e) were measured by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control.

5.3.4 Effects of EXD on protein expression of different ERs in bone

5.3.4.1 Effect of GPER antagonist and siRNA on cell viability in EXD-treated osteoblasts

The results showed that EXD, like E_2, increased the cell viability of MC3T3-E1 cells and MG-63 cells at the dosages at 0.1, 1, 10 and 100 μg/ml and from 0.1 to 100μg/ml, respectively (Figure 5.8). 1, 10, and 100 μg/ml were chosen to be the optimal doses in both cells. G15 pre-treatment for 20 minutes did not affect the cell viability of both MC3T3-E1 and MG63 cells at their basal state when compared to the control group. However, G15 pre-treatment significantly enhanced the induction by EXD on cell viability at 1μg/ml in MG-63 cells, but did not affect that in MC3T3-E1 cells (P<0.05 vs. Treatment group without G15). NT siRNA transfection did not affect the cell viability of both cells when compared to the control group. GPER siRNA transfection significantly enhanced the induction of cell viability by E_2 and EXD at 1μg/ml MG-63 cells, but did not affect that in MC3T3-E1 cells (P<0.05 vs. treatment group transfected with NT siRNA).

5.3.4.2 Effect of GPER antagonist on mRNA expression of bone markers in EXD-treated osteoblasts

In figure 5.9, Our results showed that EXD at 10 and 100 μg/ml, acted like 10^{-8}M E_2, significantly increased the mRNA expression of OCN by 1.2-fold, OPG by 1.2 fold and OPG/RANKL ratio by 1.7-fold while reduced RANKL mRNA expression by 0.7-fold in MC3T3-E1 cells (P<0.05 vs. Control). ALP mRNA expression was not altered in MC3T3-E1 cells upon treatment with EXD and E_2. Moreover, pre-treatment of MC3T3-E1 cells with G15 further uplifted OCN, OPG mRNA, and OPG/RANKL induced by 10μg/ml EXD as well as OPG mRNA stimulated by 10^{-8}M E_2 (P<0.05 vs. Treatment group without G15 pre-treatment). G15 pre-treatment did not alter the mRNA expression of ALP and RANKL in MC3T3-E1 cells. Similarly, 10^{-8}M E_2 and EXD at 10μg/ml increased the mRNA expression of OCN by 1.5-fold, OPG by 1.2-fold, and OPG/RANKL ratio by 1.5-fold, and decreased RANKL mRNA expression by 0.8-fold in MG-63 cells (P<0.05 vs. Control). Pre-treatment of MG-63 cells with G15 significantly enhanced 10^{-8}M E_2- induced OPG mRNA expression in MG-63 cells (P<0.05 vs. treatment group without G15 pre-treatment). It also tended to enhance the stimulation of OCN mRNA expression and OPG/RANKL by 10^{-8}M E_2 without statistical significance in MG-63 cells. G15 pre-treatment did not alter the mRNA expression of ALP and RANKL in MG-63 cells.

5.3.4.3 Effect of GPER siRNA on mRNA expression of bone markers in EXD-treated osteoblasts

EXD (10 and 100μg/ml) and E_2 (10^{-8}M) treatment for 24 hours significantly (P<0.05 vs. control group transfected with non-targeting siRNA) induced the mRNA expression of OCN by 1.2-fold, OPG by 1.3-fold as well as OPG/RANKL ratio by 1.7-fold while suppressed (P<0.05 vs. NT siRNA) RANKL by 0.7-fold in MC3T3-E1 cells (Figure 5.10). Both EXD and E_2 treatment did not alter the mRNA expression of ALP in MC3T3-E1 cells. Moreover, GPER knockdown led to significant (P<0.01 vs. treatment group transfected with non-targeting siRNA) increase in EXD- and E_2-induced mRNA expression of OPG, but not other bone marker expressions in MC3T3-E1 cells.

EXD (10 and 100μg/ml) and E_2 (10^{-8}M) treatment significantly (P<0.05 vs. NT siRNA) increased the mRNA expression of OCN by 1.3-fold, ALP by 1.2-fold, OPG by 1.25-fold as well as OPG/RANKL by 2-fold while decreased (P<0.05 vs. Control group transfected with non-targeting siRNA) RANKL expression by 0.6-fold in MG-63 cells. GPER knockdown could further (P<0.05 vs. treatment group transfected with non-targeting siRNA) increase the mRNA expression of OPG induced by 10μg/ml EXD, but not E_2. GPER knockdown did not alter the expression of other bone markers upon EXD and E_2 treatment in MG-63 cells.

5.3.4.4 Effect of GPER antagonist on estrogenic signaling in EXD-treated osteoblasts

Our results demonstrated that E_2 (10^{-8}M) and EXD (10μg/ml) rapidly induced the phosphorylation of Akt, ERK, ER-α (Ser167), and (Ser118) in MC3T3-E1 cells (P<0.05 vs. Control) (Figure 5.11). Pre-treatment with G15 further enhanced E_2-induced phosphorylation of ER-α (Ser118) and EXD-induced phosphorylation of ERK within 10 minutes of treatment in MC3T3-E1 cells (P <0.05 vs. treatment group without G15 pre-treatment). Similarly, E_2 (10^{-8}M) and EXD (10μg/ml) rapidly increased the phosphorylation of ERK, ER-α (Ser167) in MG-63 cells (P <0.05 vs. control). Pre-treatment with G15 further enhanced E_2-induced phosphorylation of ER-α (Ser118) and EXD-induced phosphorylation of Akt, ERK, ER-α (Ser167) within 10 minutes (P<0.05 vs. treatment group without G15 pre-treatment).

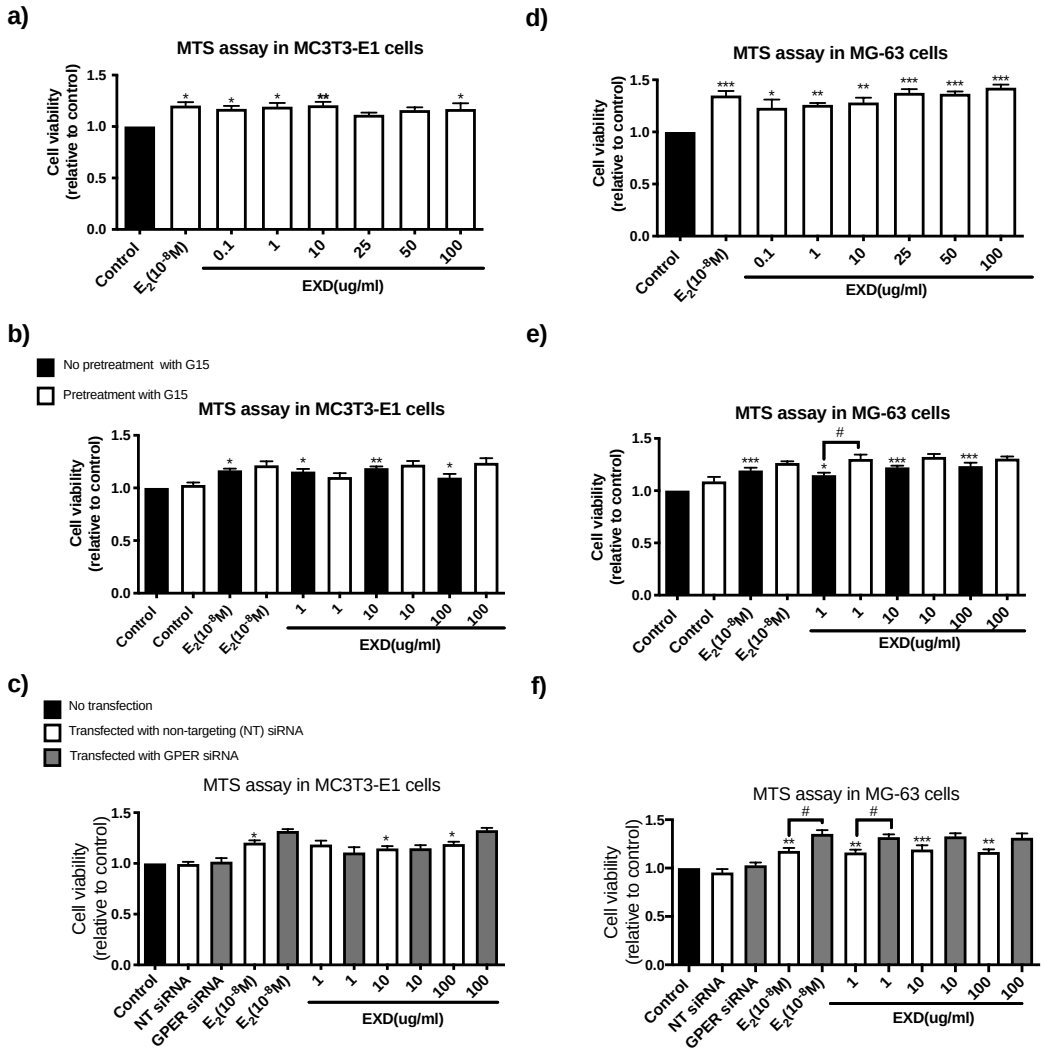


Figure 5.8 The effect of EXD on cell viability in osteoblasts with GPER knockdown or G15 pre-treatment.

MC3T3-E1 cells (a) and MG-63 cells (d) were treated with 0.1 to 100 μg/ml EXD for 24 hours to determine the optimal dosages. MC3T3-E1 cells (b-c) and MG-63 cells (e-f) were then transfected with non-targeting siRNA(NT), GPER siRNA at desired dosages or pre-treated with G15(10^{-6}M) before the 24-hour treatment with vehicle, E_2 or EXD. Cell viability was assessed by MTS assay. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control (a,b,d and e) or NT siRNA (c and f); $^{\#}$ $P<0.05$ vs. treatment group transfected with NT siRNA(c and f) or pre-treated with G15 (d and e).

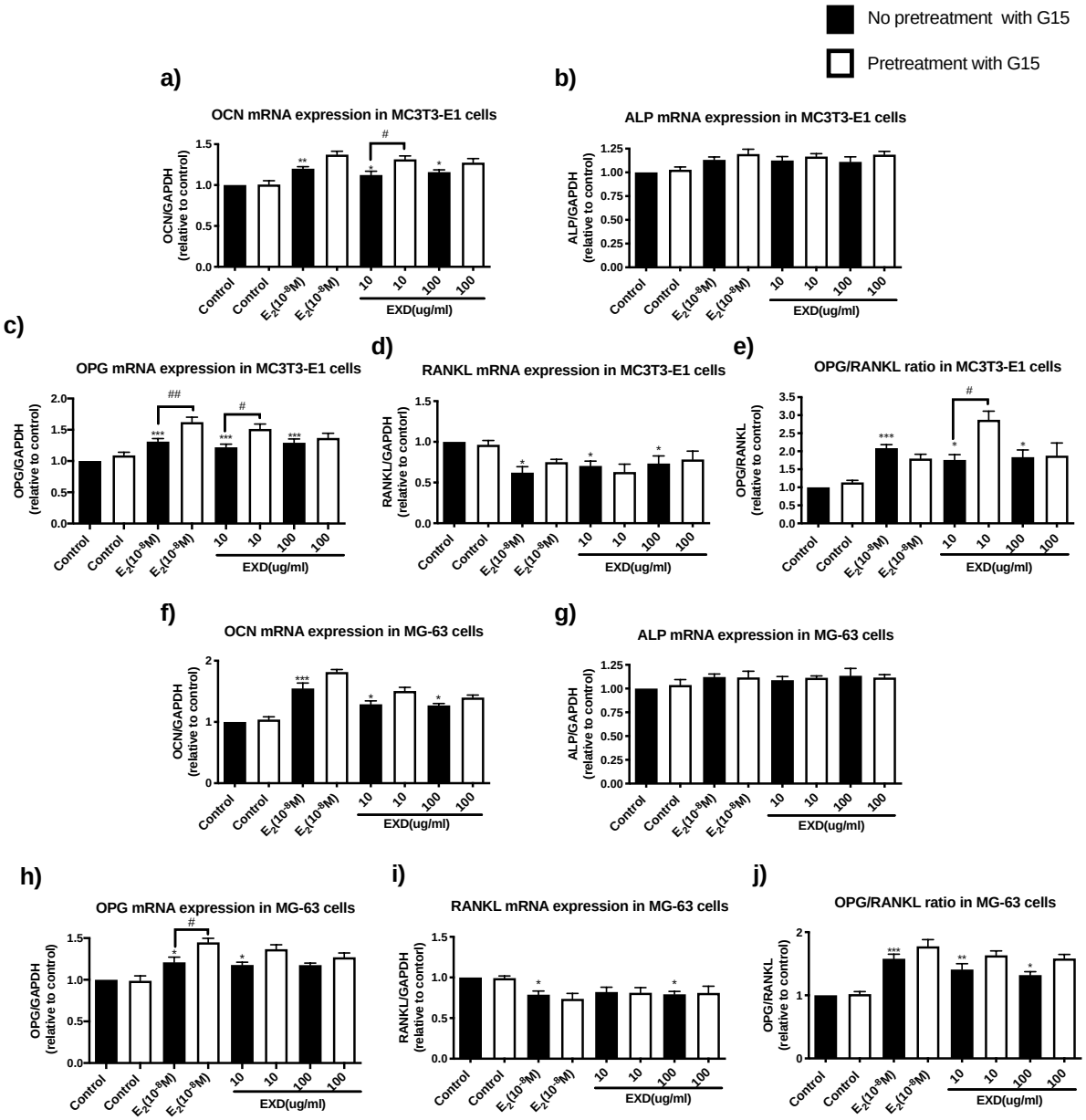


Figure 5.9 Effect of GPER antagonist on mRNA expressions of bone markers induced by EXD in osteoblasts.

MC3T3-E1 cells and MG-63 cells were pre-treated with GPER antagonist, G15 before treatment with vehicle, E_2, or EXD for 24 hours. mRNA was extracted by using Trizol reagent. mRNA expressions of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio in MC3T3-E1 cells (a-e) and MG-63 cells (f-j) were amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}$ $P<0.05$ and $^{\#\#}$ $P<0.01$ vs. treatment group pre-treated with G15.

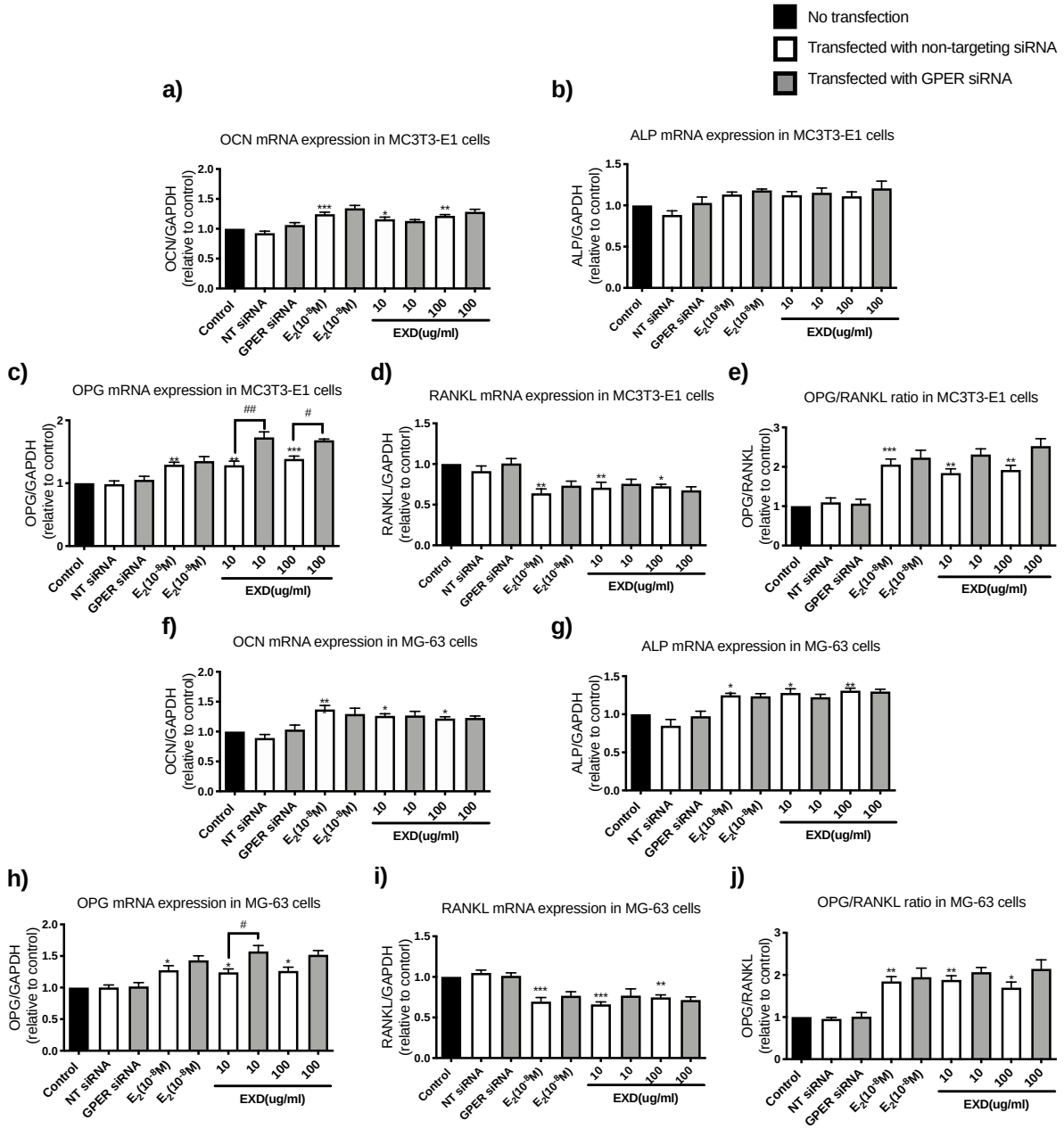


Figure 5.10 Effect of GPER knockdown on mRNA expressions of bone markers induced by EXD in osteoblasts.

MC3T3-E1 cells and MG-63 cells were transfected with GPER siRNA before treatment with vehicle, E_2, or EXD for 24 hours. mRNA was extracted by using Trizol reagent. mRNA expression of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio in MC3T3-E1 cells (a-e) and MG-63 cells (f-j) were amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. NT siRNA; $^{\#}$ $P<0.05$ and $^{\#\#}$ $P<0.01$ vs. treatment group transfected with NT siRNA.

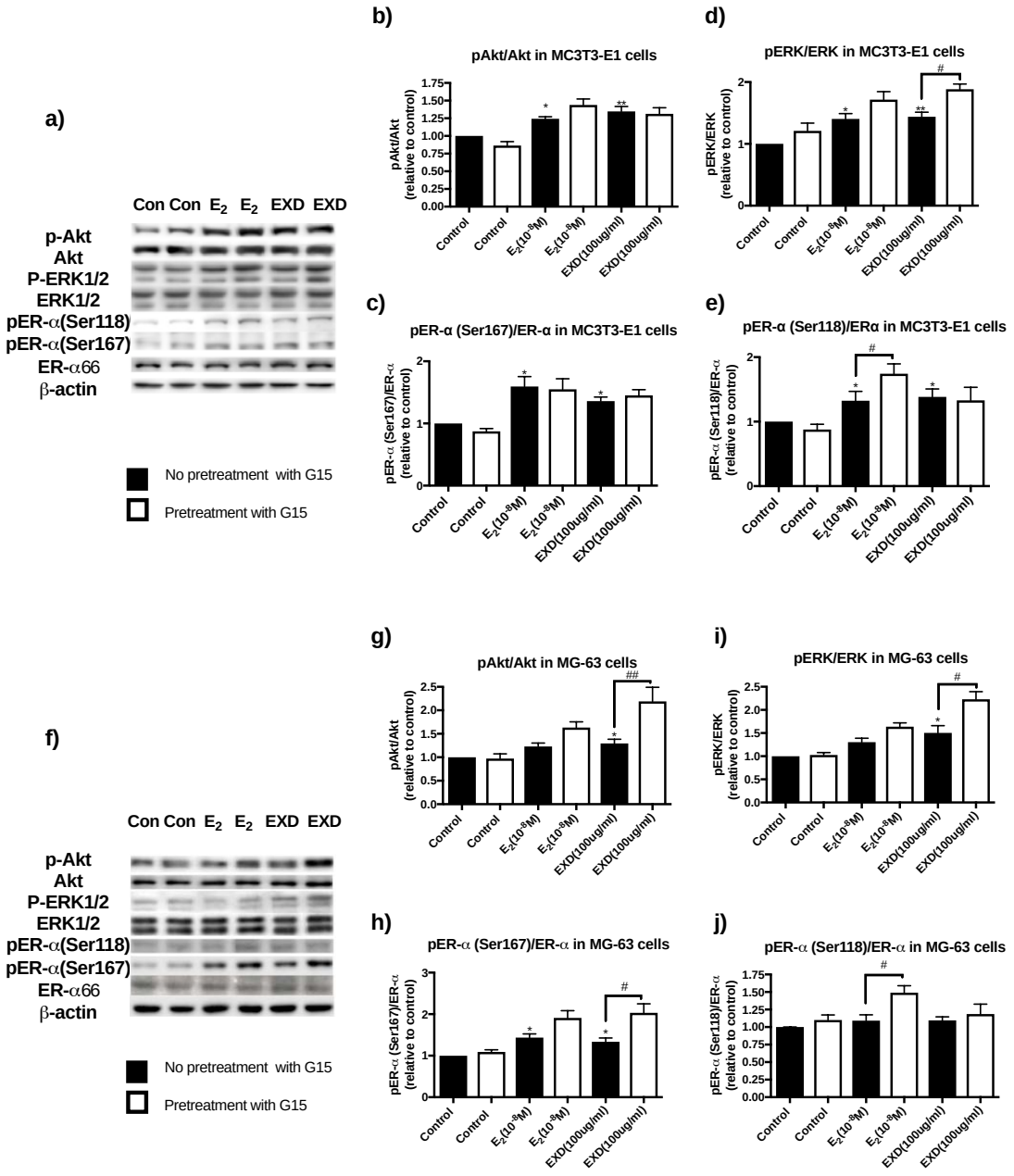


Figure 5.11 The effects of G15 pre-treatment on rapid signaling induced by EXD in osteoblasts.

MC3T3-E1 cells (a-e) and MG-63 cells (f-j) were pre-treated with G15(10^{-6}M) for 20 minutes before treatment with vehicle, E_2, or EXD for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER-α (Ser167), pER-α (Ser118), and ER-α66 were detected by western blotting in both cells. The protein expression level was shown as the ratio of the target protein to β-actin. The figures are the representatives of three independent experiments in MC3T3-E1 cells and MG-63 cells. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}$ $P<0.05$ and $^{\#\#}$ $P<0.05$ vs. treatment group without G15 pre-treatment.

5.3.4.5 **Effect of GPER siRNA on estrogenic signaling in EXD-treated osteoblasts**

Knockdown of GPER in MC3T3-E1 cells did not alter E_2- induced phosphorylation of Akt, ERK, ER-α (Ser167) and (Ser118) while GPER knockout further (P<0.05 vs. treatment group transfected with NT siRNA) enhanced EXD-induced phosphorylation of Akt and ER-α (Ser167) within 10 minutes in MC3T3-E1 cells (Figure 5.12). GPER knockdown did not alter EXD- or E_2-induced phosphorylation of ERK and ER-α (Ser118) in MC3T3-E1 cells. Moreover, our results demonstrated that GPER knockdown further enhanced E_2-induced phosphorylation of Akt and EXD-induced phosphorylation of Akt and ERK in MG-63 cells (P<0.05 vs. treatment group transfected with NT siRNA). GPER knockdown did not alter EXD- or E_2-induced activation of ER-α (Ser118) and (Ser167) in MG-63 cells.

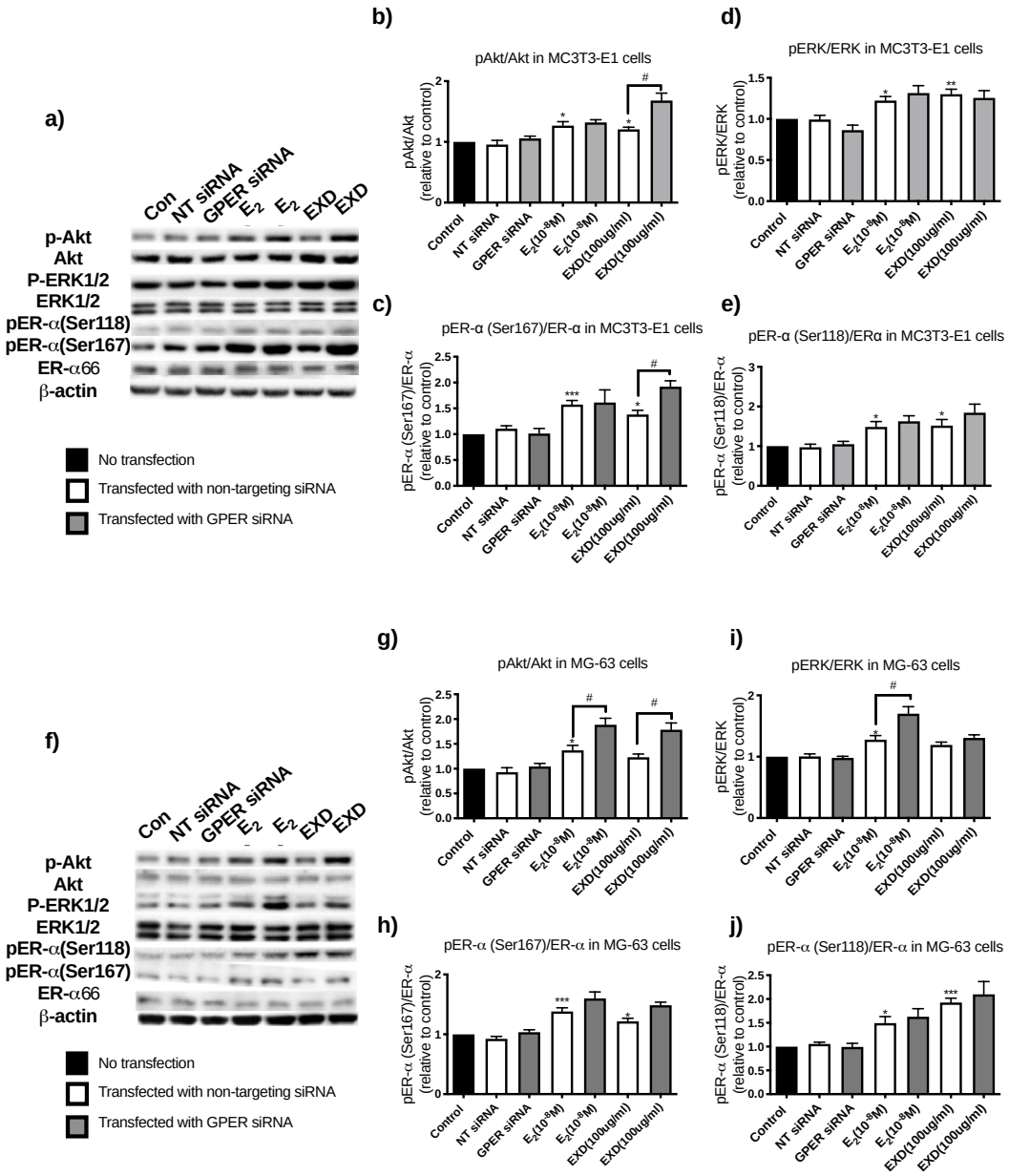


Figure 5.12 The effect of GPER knockdown on rapid ER signaling induced by EXD in osteoblasts.

MC3T3-E1 cells (a-e) and MG-63 (f-j) cells were transfected with non-targeting siRNA(NT) and GPER siRNA at desired dosages before treatment with vehicle, E_2, or EXD for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER-α (Ser167), pER-α (Ser118), and ER-α66 were studied by western blotting. The figures are the representatives of three independent experiments. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ and *** $P<0.001$ vs. NT siRNA; # $p<0.05$ vs. treatment group transfected with NT siRNA.

5.3.5 Characterization of the role of ER-α36 in bone protective effect of EXD

5.3.5.1 Effect of ER-α36 overexpression on cell viability in EXD-treated osteoblasts

MC3T3-E1 and MG-63 cells were transfected with CMV-36 or control vector before treatment with EXD for 24-hour. As expected (Figure 5.13), E_2 (10^{-8}M) and EXD significantly increased cell viability by 1.2-fold in MC3T3-E1 (1 to 100μg/ml) and MG-63 cells (10 and 100μg/ml). Overexpression of ER-α36 suppressed the increase in cell viability by E_2 and EXD (10 and 100μg/ml) in both cells (P<0.05 vs. treatment group transfected with CMV).

5.3.5.2 Effect of ER-α36 overexpression on mRNA expression of bone markers in EXD-treated osteoblasts

Overexpression of ER-α36 significantly enhanced EXD-induced mRNA expression of OPG and OPG/RANKL ratio at 100 μg/ml (P<0.05 vs. Treatment group transfected with CMV) (Figure 5.13). Moreover, the mRNA expression of ALP appeared to be reduced when MC3T3-E1 cells were transfected with the ER-α36 expression vector, but the changes did not reach statistical significance. In addition, overexpression of ER-α36 in MG-63 cells reversed the stimulatory effects of E_2 or 100 μg/ml EXD on mRNA expression of OPG and OPG/RANKL ratio, as well as the inhibitory effects on RANKL mRNA expression (P<0.05 vs. treatment group transfected with CMV). However, overexpression of ER-α36 did alter E_{2-} and EXD-stimulated changes in ALP and OCN mRNA expression in MG-63 cells.

5.3.5.3 Effect of ER-α36 overexpression on estrogenic signaling in EXD- treated osteoblasts

Our results demonstrated that ER-α36 overexpression significantly decreased the phosphorylation of Akt by EXD in MC3T3-E1 cells as well as phosphorylation of ER-α (Ser167) and (Ser118) induced by E_2 or EXD (P<0.05 vs. Treatment group transfected with CMV) (Figure 5.14). The suppressive effects of ER-α36 overexpression on phosphorylation of ERK induced by EXD in MC3T3-E1 cells did not reach statistical significance. Similarly, ER-α36 overexpression reversed E_2 or EXD-induced phosphorylation of ERK, ER-α (Ser167), and (Ser118) in MG-63 cells (P<0.05 vs. treatment group transfected with CMV). The suppressive effects of ER-α36 overexpression on phosphorylation of Akt and ERK induced by E_2 or EXD in MG-63 cells did not reach statistical significance.

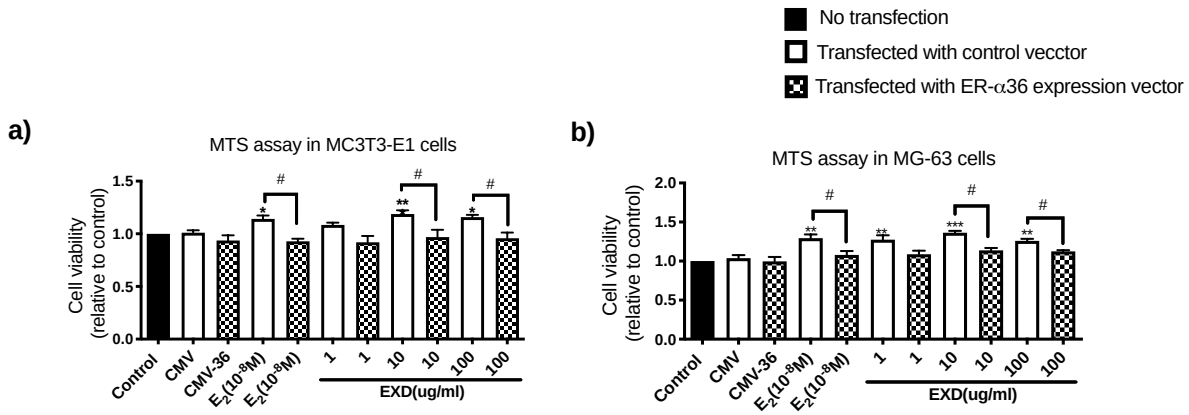


Figure 5.13 The effect of EXD on cell viability in osteoblasts overexpressed with ER-α36. MC3T3-E1 (a) and MG-63 cells (b) were transfected with CMV and CMV-36 plasmid at optimal doses before treatment with vehicle, E_2, or EXD for 24 hours. Cell viability was assessed by MTS assay. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ vs. Treatment group transfected with CMV.

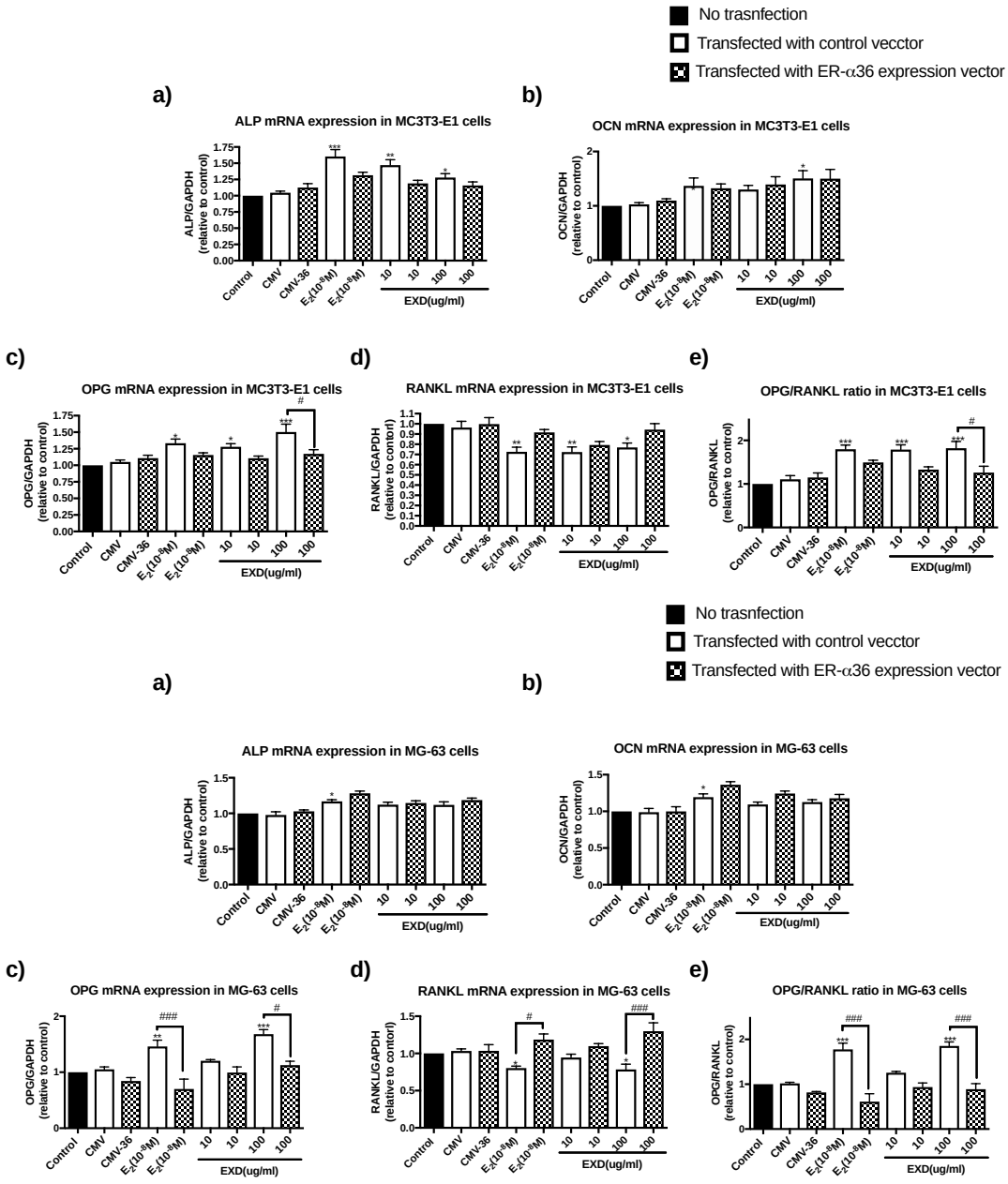


Figure 5.14 Effect of ER-α36 overexpression on mRNA expressions of bone markers induced by EXD in osteoblasts.

MC3T3-E1 (a-e) and MG-63 cells (f-j) were treated with vehicle, E_2, or EXD for 24 hours in phenol red-free DMEM containing 1% cs-FBS. mRNA was extracted by using Trizol reagent. mRNA expression of OCN, ALP, OPG, RANKL, and OPG/RANKL ratio were amplified by real-time PCR. The mRNA expression level is shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ vs. Control ; $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.001$ vs. Treatment group transfected with CMV.

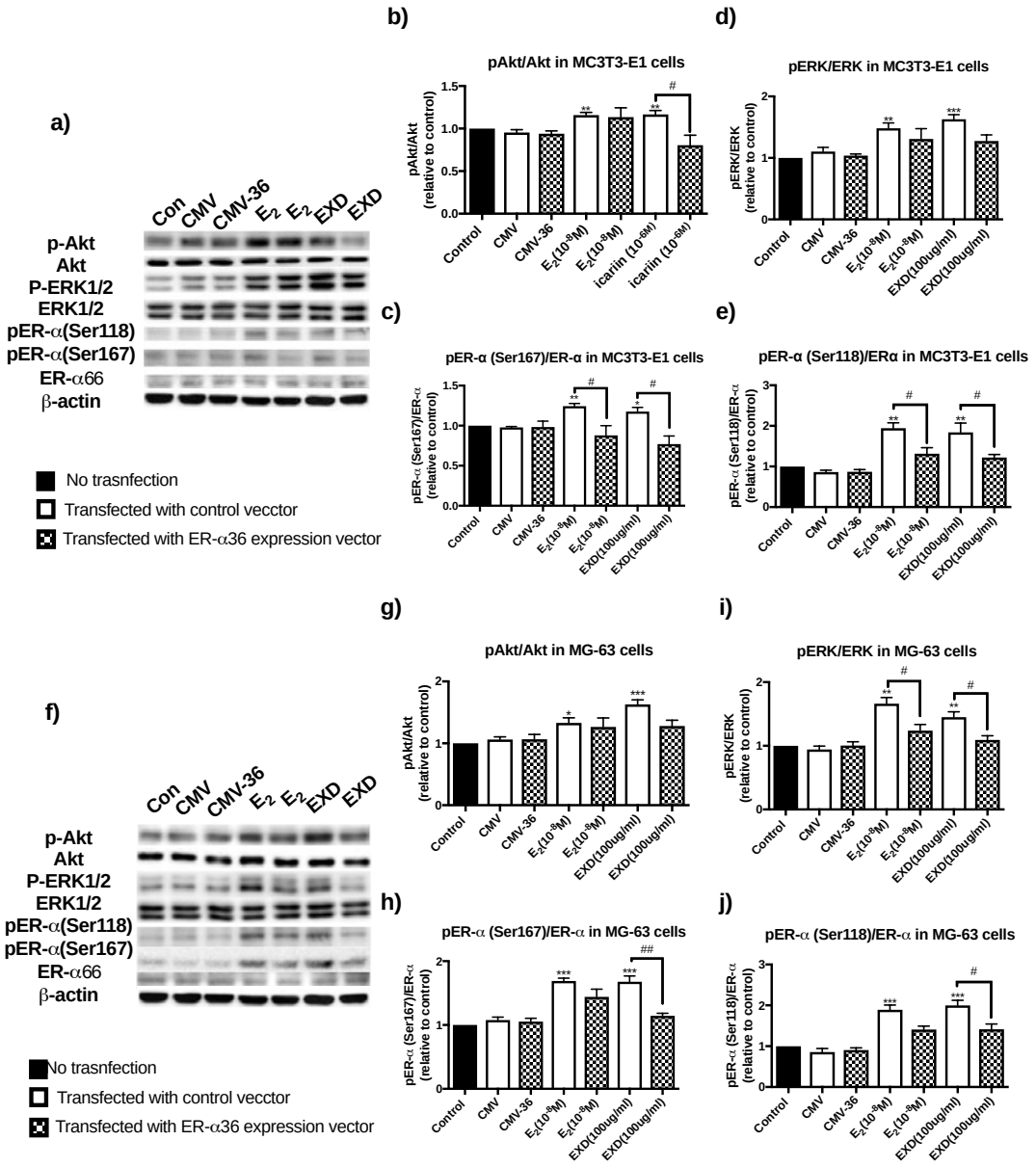


Figure 5.15 The effect of ER-α36 overexpression on rapid signaling induced by EXD in osteoblasts.

MC3T3-E1 (a-e) and MG-63 cells (f-j) were transfected with CMV or CMV-36 at desired dosages before treatment with vehicle, E_2, or EXD for 10 minutes. Protein expression of different signaling molecules, pAkt, Akt, pERK, ERK, pER-α (Ser167), pER-α (Ser118), and ER-α66 were studied by western blotting. The figures are the representatives of three independent experiments. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. Control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. treatment group transfected with CMV.

5.4 Discussion

In this chapter, we have explicated that EXD is an alternative approach managing postmenopausal osteoporosis with high efficacy and safety, probably via hormonal regulation. *In vitro* study revealed that such effects of EXD might also occur via the regulation of expression of different ERs, i.e. increasing the expression of ER-α66 while suppressing the expression of two novel membrane estrogen receptor ER-α36 and GPER. Such regulation might facilitate ER-α66-induced rapid estrogen signaling for cell proliferation and bone remodelling.

EXD could exert bone protection without uterotrophic effect in OVX rats, probably via hormone regulation.

Long term administration of EXD could prevent bone deterioration caused by OVX in rats. Our study showed that EXD worked well in restoring trabecular bone of long bone as well as lumbar spine. Such bone protection was also reported in the clinical and preclinical studies (Xue et al., 2011), suggesting EXD can be used for treatment of postmenopausal osteoporosis. It is worth to note that the upregulation of serum osteocalcin in OVX rats might account for the increased body weight in OVX rats via its endocrine action in adipogenesis and lipid accumulation. Previous study suggested that most osteocalcin secreted from osteoblast is incorporated into bone extracellular matrix, but small amount of osteocalcin would undergo decarboxylation and released into circulation (J. Wei & Karsenty, 2015). Circulating osteocalcin could exert endocrine action on putative receptor Gprc6a in adipocytes and increase the secretion of adiponectin. Adiponectin was reported to promote adipocyte differentiation and lipid accumulation(O'Connor & Durack, 2017). Also, circulating osteocalcin was also found to increase the insulin sensitivity of adipocyte, in turn, decrease the rate of lipolysis in adipose tissues, lower the plasma fatty acid level and increase the uptake of triglycerides from the blood into adipose tissue and muscles (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011). In clinical study, postmenopausal women showed to have higher serum osteocalcin level which may due to the release of osteocalcin from hydroxyapatite in bone matrix during osteoporosis. Free osteocalcin and increase in FSH in postmenopausal women could lead to secretion of serum adiponectin. Our present results in OVX rats are in line with previous study that OVX in rats, mimicking postmenopausal situation, had elevated serum FSH and osteocalcin level. Thus, serum osteocalcin level previously considered as a bone formation marker might not fully reflect osteoblastogenesis, it might be an indicator of adipogenesis in rats instead. Our results supported that EXD could restore the serum osteocalcin level as well as body weight when compared to osteoporotic OVX rats. It might indicate EXD could regulate

adipogenesis and hence reduce body weight via downregulating serum osteocalcin in OVX rat. We demonstrated that EXD could also restore serum estrogen level, but suppressed serum FSH and LH level in OVX. These results are line with other studies that EXD could regulate hormone secretion by modulation of the hypothalamic-pituitary axis. Study by Sze et al suggested that EXD could stimulate aromatase activity and produce more estrogen in ovary, liver, and fats (Gonzalez-Robayna et al., 2000; Sze et al., 2009), thereby maintaining bone formation. The increase in serum FSH level is found to begin even before the decrease in estrogen and this increased FSH is accompanied with a boost in bone markers and decrease in adipogeneses via suppressing serum adiponectin . In agreement with the report, a dramatic elevation was also observed in the present study in OVX rats in which all bone-protective agents, including estradiol, EXD and their combinations, suppressed the increase in FSH level. The changes in BMD and bone turnover markers appeared to be associated more likely with the changes in circulating FSH than estradiol in OVX rats (Chin, 2018). However, it is unclear if LH also plays a role in mediating the actions of bone protective effects of TCM. Although EXD could increase the serum estrogen level in OVX rats, EXD did not stimulate the growth of the uterus, as indicated by the uterus index and epithelial thickening of the uterus in OVX rats. The tissue selectivity by EXD in bone health may be due to the ratio of different ERs being expressed and activated in bone by EXD. Thereby, different estrogenic responses mediated by ERs appear in various tissues.

EXD exerts bone protection by activating ER-α66 mediating rapid estrogenic signaling

Our results showed that the estrogenic bone protective effects of EXD is likely mediated by ER-α66. *In vivo* study suggests a feedback mechanism on ER-α66 toward OVX-induced low level of E_2 production. It might promote the tissue sensitivity of E_2 by providing more ERs in tibia head. *In vitro* study further studied the regulation of the expression of different ERs by EXD. EXD could increase the protein expression of ER-α66 as well as phosphorylation of ER-α (Ser118) and (Ser167) within 10 minutes of treatment in both MC3T3-E1 cells and MG-63 cells. Previously, rapid phosphorylation of ER-α66 by ERK1/2 was suggested to be involved in mediating the actions of mechanical strain, cell proliferation, as well as differentiation in bone cells. ICI 182,170, an ER antagonist, could block EXD-induced increase in cell viability in rat osteoblast-like UMR-106 cells (K. C. Wong et al., 2014). This indicated ER-α66 is required in the estrogenic bone protective effects as well as the activation of rapid signaling by EXD.

Unlike ER-α66, ER-α36 seems to be a negative regulator in EXD action in bone.

Our study showed that E_2 and EXD treatment could suppress ER-α36 mRNA and protein expression in both MC3T3-E1 cells and MG-63 cells. Such observation is in agreement with those reported in human osteoblasts in which the addition of E_2 could reduce ER-α36 expression while increase ER-α66 expression in osteoblasts harvested from postmenopausal women (Xie et al., 2011). Moreover, the differential regulation of the expression of these two receptors was observed in human breast cancer MCF-7, H3396 cells (L. Kang & Z.-Y. Wang, 2010) as well as osteoblasts harvested from postmenopausal women (Xie et al., 2011). These results suggest that ER-α36 might have a different role from ER-α66 in mediating estrogenic bone protective effects.

We then utilized ER-α36-overexpressed cells to study its role in bone protective effects of EXD. Overexpression of ER-α36 could abolish the effects of E_2 or EXD on cell viability and mRNA OPG/RANKL ratio in MC3T3-E1 cells and MG-63 cells. Such effects of ER-α36 were similar to those reported by Wang et al. (Xie et al., 2011) in which knockdown of ER-α36 further increased E_2-induced ALP and OCN mRNA expression in osteoblasts isolated from postmenopausal women. Interestingly, ER-α36 was reported to strongly inhibit the transcriptional activities of ER-α66 by retaining ER-α66 in the cytoplasm via formation of heterodimer ER-α66/ER-α36 (Z. Y. Wang & Yin, 2015). Thus, the suppression of ER-α36 expression by EXD and E_2 in both cells might result in inhibiting the crosstalk between ER-α36 and ER-α66 for regulation of OPG/RANKL mRNA expression. Although overexpression of ER-α36 in our *in vitro* study did not alter the ALP and OCN mRNA expression in cell lines, the results here indicated ER-α36 might not favour cell proliferation and bone remodelling by EXD or E_2.

Our results showed that ER-α36 seems to be a suppressor in the Akt pathway and phosphorylation of ER-α (Ser118) and (Ser167) in MC3T3-E1 cells upon treatment with EXD for 10 minutes. Whereas, ERK pathway and phosphorylation of ER-α (Ser118) and (Ser167) in MG-63 cells were suppressed upon treatment with EXD or E_2 for 10 minutes. The ability of ER-α36 to activate ERK was reported by others as ERK could directly bind to ER-α36 that lead either activation or deactivation of the MAPK/ERK pathway (Omarjee et al., 2017). These results suggest the involvement of ER-α36 was different from that of ER-α66 in bone remodelling of pre-osteoblast and osteoblast.

Taken together, EXD and E_2 treatment appeared to downregulate ER-α36 expression to prevent the inhibitory effects of ER-α36 on ER-α66-induced cell viability, bone remodelling, and rapid estrogenic signaling in osteoblasts.

<u>GPER seems to be a negative regulator in the bone protection of EXD</u>

In vitro study showed that both EXD and E_2 could suppress the protein expression of GPER, similar to ER-α36, in both MC3T3-E1 cells and MG-63 cells upon treatment for 24 hours. The results indicated that EXD and E_2 recruited GPER to mediate their bone protective effects, and GPER was also inversely regulated when compared to ER-α66 expression. These results are in consistence with other studies that introducing GPER could induce endogenous ER-α36 in GPER non-expressing HEK293 cells, while knockdown of GPER could lead to downregulation of ER-α36 in breast cancer SK-BR-3 cells (Lianguo Kang et al., 2010).

The role of GPER in bone remodelling is still controversial. Some studies proposed that GPER knockout (KO) mice were found to lack normal regulation of the epiphyseal growth plate and estrogen-mediated insulin-secretion (Mårtensson et al., 2009). A study by Windahl et al. also showed that estrogen replacement was unable to reduce longitudinal bone growth in OVX GPER KO mice (Windahl et al., 2009). Oppositely, GPER-deficient male mice was shown to exhibit increase in femur size, BMD, and trabecular bone volume when compared to wide-type mice (Ford et al., 2011). Other studies also mentioned that GPER knockout could increase the biomechanical properties, bending stiffness as well as BMD in a 6-week mouse femur fracture model, implying the inhibitory effect of GPER on bone repair (J. et al., 2012). *In vitro* study also elucidated that GPER negatively regulated estrogenic-differentiation in murine BMSCs by suppressing estrogenic gene expression, such as BMP-2, osteocalcin, and ALP, as well as mineralization (J. Zhong et al., 2019). Thus, to study the role of GPER in the bone remodelling of EXD and E_2, we utilized GPER-knockdown or G15 (a GPER specific blocker)-pre-treated MC3T3-E1 cells and MG-63 cells. Blocking GPER by G15 pre-treatment abolished its inhibitory effects on EXD-induced mRNA expression of OCN, OPG as well as OPG/RANKL ratio, and EXD- and E_2-induced OPG mRNA expression in both cells. Likewise, GPER knockdown could further enhance EXD-induced cell viability and mRNA expression of OPG in both cells, and E_2-induced in MG-63 cells. It indicates that GPER involved in the bone protective effect of EXD as a negative regulator on cell viability and bone formation. Indeed, we are the first to report that GPER is a negative regulator in bone remodelling that mediates the actions of EXD.

GPER is a transmembrane protein that could be transported to the cell membrane from the endoplasmic reticulum upon the activation signal. High binding affinity to E_2 of extracellular GPER makes it a responder in nongenomic signaling. Our results showed that phosphorylation of Akt and ERK, as well as ER-α (Ser118) and (Ser167) induced by EXD or E_2, were enhanced when GPER is being blocked or knockdown. It indicated that GPER played an inhibitory role in Akt and ERK pathway as well as ER-α66 phosphorylation in osteoblastic cells in response

to EXD. It is widely studied that PI3K/Akt and ERK/MAPK signaling pathways play important roles in regulating cell proliferation, ALP activity, calcium accumulation, and mRNA expression of OCN and OPG in bone remodelling (Xi et al., 2015). Collectively, EXD and E_2 downregulated GPER expression and suppressed rapid estrogenic signaling, thereby reducing its inhibitory effect on osteoblastogenesis.

In summary, EXD is an alternative approach for managing postmenopausal osteoporosis with efficacy and safety. 12-week EXD treatment could exert bone protective effect in female OVX SD rats by modulating hormone secretion, bone turnover, as well as maintaining bone properties without uterotrophic effects. ER-α66-mediated estrogenic pathway might account for such effect by EXD. ER-α36 and GPER are participated in the bone protection of EXD as negative feedback suppressors (Figure 5.15). However, the unique signal transductions of ER-α36 and GPER are difficult to study in ERs-positive cells because of physical and functional interaction amongst ERs. Also, ER-α36 and GPER seem to mediate estrogen induction in both ER-α66-positive and ER-α66-negative cells. Their action could be ER-α66-dependent or independent. In other words, the activation of non-genomic responses in intact cells does not distinguish between ER-α isoforms. A study on the downstream signaling by ER-α36 alone in EXD or E_2- treated ER-α66-deficient osteoblasts is required in order to study the distinct role of novel estrogen receptors in the bone protective effects of EXD.

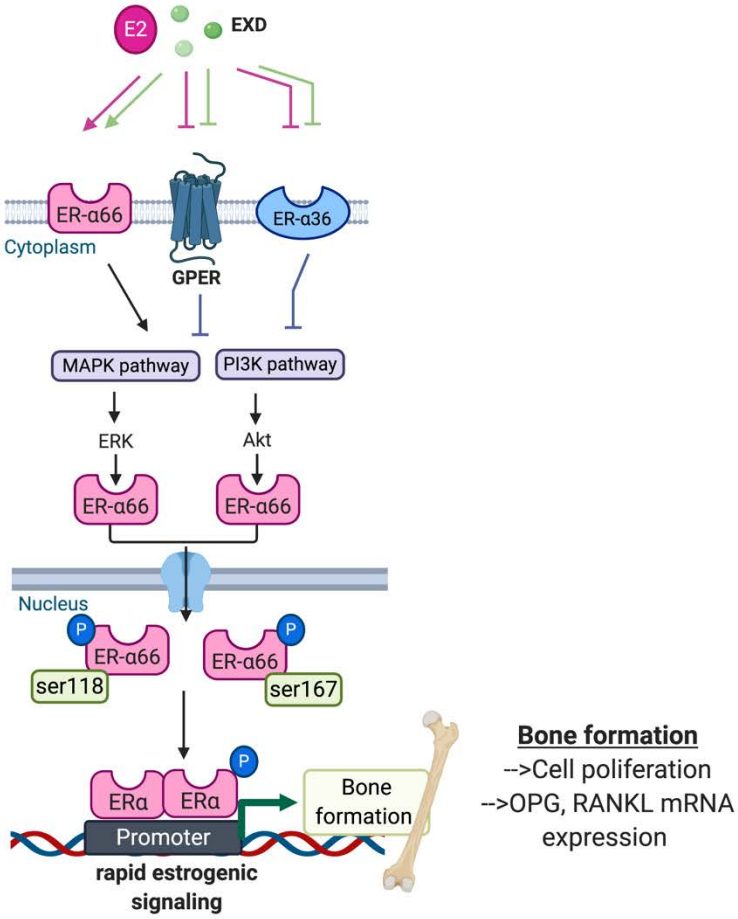


Figure 5.16 Schematic mechanisms illustrating the involvement of ERs in response to E_2 and EXD in bone.

E_2 and EXD exert osteoblastogenesis by regulation of ER-α36, ER-α66, and GPER. E_2 and icariin could suppress the inhibitory effects of ER-α36 and GPER in rapid estrogen signaling as well as reducing inhibitory effects of ER-α36 and GPER on osteoblastogenesis induced by EXD and E_2.

Chapter 6. Characterization of ER-α66-dependency of the bone protective effects of icariin and EXD

6.1 Introduction

Postmenopausal osteoporosis is mainly caused by reduction of estrogen production in ovary. Many therapies for maintaining bone health in menopausal women are targeting estrogen receptors and estrogen signaling (Ji & Yu, 2015). Recent studies indicated that classical ERs, namely ER-α66 and estrogen receptor β (ER-β), are not the sole estrogen responders. Two novel ERs, estrogen receptor α 36 (ER-α36) and G-protein coupled estrogen receptor (GPER), were discovered to mediate mainly nongenomic estrogen signaling in both ER-α66-negative or positive cells (Z. Y. Wang & Yin, 2015). Besides, a Traditional Chinese medicine (TCM) formula, Er-xian decoction (EXD), and a phytoestrogen, icariin, were clinically used to manage menopausal syndrome presumably via activation of ERs. Tissue-selective estrogenic properties of EXD and icariin contributed to their safety and efficacy in managing postmenopausal osteoporosis without inducing severe side effects (J.-Y. Li et al., 2017; Y. Wang et al., 2019; M. S. Wong & Zhang, 2013). In previous chapters, we have demonstrated that EXD and icariin might recruit ER-α36 and GPER in estrogen signaling activation and bone formation, likely as negative regulators of ER-α66 via physical interactions.

The unique signal transductions of ER-α36 or GPER are difficult to study in ERs-positive cells, especially ER-α66-positive condition, as their actions might be affected by the complicated physical and functional interactions amongst ERs as illustrated in osteoblasts in the previous chapters. To address this problem in this chapter, we have utilized female osteocalcin-drive osteoblast-specific ERα knockout (pOC-ERαKO) mice to study the distinct roles of ER-α36 and GPER in mediating ER-α66-independent icariin- or EXD-induced bone protection. pOC-ERαKO mice were previously generated by Dr. Marjolein van der Meulen at Cornell University. Mice were generated (Figure 6.1) by breeding mice with exon 3 of the DNA binding domain of the ERα gene (Esr1) flanked by loxP sequences, $ERα^{fl/fl}$, to mice containing a transgene encoding Cre recombinase driven by the human osteocalcin promoter, OC-Cre (Melville et al., 2014).

Taking advantage of pOC-ERαKO mice, the importance of ER-α66 in regulation of bone properties was explicated. Basal expression of different ERs protein expression in osteoblast was compared between littermate control (LC) and pOC-ERαKO mice. The effects of E_2, icariin or EXD on osteoblast viability, rapid phosphorylation of ER-α(Ser118), and ERK were also studied in osteoblast isolated from LC or pOC-ERαKO mice. It is hoped that this study could provide more information on the particular roles of these ERs in bone remodelling mediated by E_2, icariin or EXD.

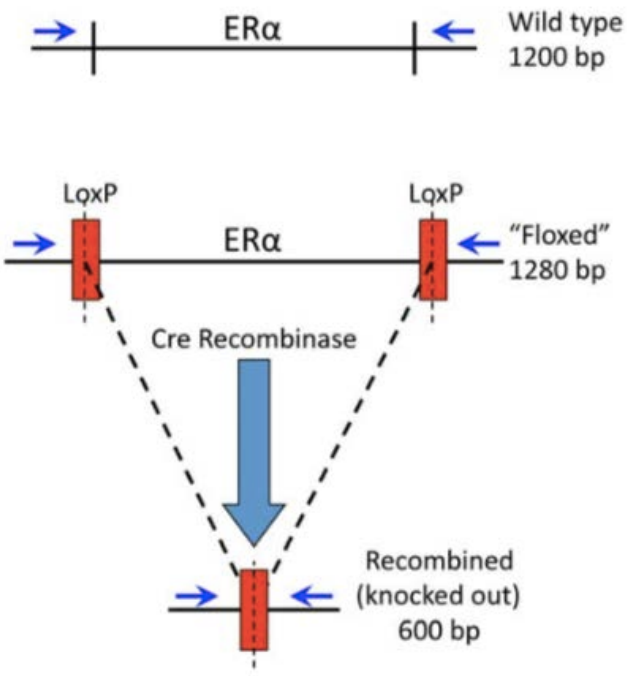


Figure 6.1 Schematic diagram of osteocalcin-drive osteoblast-specific ERα knockout mice.

The DNA sequences coding for exon 3 of the DNA binding domain of the ERα gene have 1200bp as indicated as wild type. Two LoxP sequences were inserted upstream and downstream of this sequence. This floxed sequence has 1280bp. With the addition of a transgene encoding Cre recombinase driven by the human osteocalcin promoter (OC-Cre), the floxed DNA sequences coding of exon 3 were cleaved by osteoblast-specific Cre recombinase. (Adapted from Cheryl Minges, 2011)

6.2 Methodology

6.2.1 Breeding and genotyping

OC-Cre mice were mated with ERα$^{fl/fl}$ (a gift from Dr. ven der Meulen at Cornell University) to obtain heterozygous mice, OC-Cre; ERα$^{fl/+}$. Female and male OC- Cre; ERα$^{fl/+}$ were mated to produce female osteoblast-specific ERα knockout mice, OC- Cre; ERα$^{fl/fl}$ (pOC-ERαKO) and wide-type as littermate control (LC). Mouse genotyping were conducted by lysed tail PCR using the primers: 5′-CAAATAGCCCTGGCAGAT-3′ (forward) and 5′-TGATACAAGGGACATCTTCC-3′ (reverse) to detect the Cre transgene, whereas the floxed ERα gene was detected using the primers: 5′-TGGGTTGCCCGATAACAATAA-3’(forward) and 5′- AAGAGATGTAGGGCGGGAAAAG-3′ (reverse) (29). Twelve-week-old LC and pOC-ERαKO female mice were sacrificed.

6.2.2 *In vivo*

6.2.2.1 Sample collection

At sacrifice, bone from mice was collected for subsequent analysis. Changes in bone mineral density (BMD) and microarchitecture in left legs and vertebra were analyzed by micro-CT. Right leg, lilac crest, and upper arms were sterilely isolated, and bone marrow stromal cells (BMSCs) and osteoblasts were collected and cultured in osteogenic medium to study the *ex vivo* effects of icariin and E_2 on osteoblastogenesis. Femur and tibia length were measured before MicroCT analysis.

6.2.2.2 MicroCT

Bone properties of trabecular bone at proximal tibia and distal femur, as well as a lumbar vertebra (L4), were determined in the present study. Scans were performed at high resolution (10.5μm) and using the energy of 70kVp, intensity of 114μA, with an integration time of 30ms. For left proximal tibia and distal femur, 100 μCT slices were acquired from the metaphyseal growth plate to metaphysics, in which the volume of interest was contoured from 50 serial of slices, corresponding to a 0.525mm region, for evaluation. The threshold for contouring volume of interest of trabecular bones is based on the adaptive method, in which the contoured images were matched with the grayscale of the background image. The threshold values for contouring trabecular bones were 375. Evaluation Program v6.0 (Scanco Medical, Switzerland) generated bone biological parameters, including bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular bone number (Tb.N, 1/mm), trabecular bone thickness (Tb.Th, mm), trabecular bone separation (Tb.Sp, mm), connectivity density (Conn.D, 1/mm^3)

and structure model index (SMI). Morphological figures of the processed volume of interest (VOI) images were generated for a three-dimension figure presentation. As for cortical bone, the bone at the midshaft of the femur and tibia were traced using the same scanning condition as trabecular bone and 2D analysis was conducted on a single section to obtain total cross-sectional area (Tt.Ar, mm^2), cortical area (Ct.Ar, mm^2), marrow area (Ma.Ar, mm^2), cortical thickness (Ct.Th, mm) and cortical area fraction (Ct.Ar/Tt.Ar).

6.2.2.3 **Real-time PCR**

The mRNA of bone formation markers (ALP and OPG), bone resorption markers (RANKL), as well as ERs (ER-α66 and GPER), were measured in tibia head of right leg by real-time PCR. Tibia heads were immersed with 100μl Trizol reagent (Thermo Fisher Scientific, Rockford, IL, United States, Cat#15596018) in Precellys Tissue grinding CKMix50 lysis kit (Bertin Technologies, France, Cat#P000939-LYSK0-A) at 4ºC. Tibia heads in lysis kits were homogenized in Precellys Evolution and Cryolys (Bertin Technologies, France) and homogenized for 10 seconds with a 10-second interval for six times. Procedures of reverse transcription, real-time PCR, and primer sequences have been illustrated in Chapter 3 and 4.

6.2.3 **Ex vivo**

6.2.3.1 **Isolation of osteoblasts and BMSCs**

Right leg, iliac crest as well as upper arms were collected in Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Rockford, IL, United States, Cat#14025076). Epiphyses were cut off to expose bone marrow, which was then be flashed out by a 5ml syringe 21Gneedle with 5ml standard culture medium into a sterile 60mm petri dish. Standard culture medium contains Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Rockville, MD, United States, Cat#12800-017) with 1% penicillin and streptomycin (Life Technologies, Rockville, MD, United States, Cat# 15140-122) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah, United States, Cat# SV30160.03) and 1% Fungizone (Thermo Fisher Scientific, Rockford, IL, United States, Cat# 15290026). The cell suspension was obtained by passing the flushed bone marrow through a 25G needle into a 15ml tube. After centrifugation at 500rpm for 5minutes, the supernatant is discarded, and the pellet was resuspended in 15ml of standard culture medium in 100mm petri dish. BMSCs were ready to use after seven days. Clean diaphysis was then subjected to osteoblast isolation. It was cut into little pieces of 1-2mm^2 using scissors. After washes with PBS, bone pieces were incubated in 1ml 0.25% trypsin at 37ºC for 10 minutes, followed by incubation in 4ml collagenase II solution (2mg/ml) solution at 37ºC in shaking water bath in order to remove all remaining soft

tissue and adherent cells for 2 hours. Rinsing the bone pieces 3 times with standard culture medium and transfer the bone pieces to 60mm dish containing 5ml medium. Osteoblast migrated from the bone chips after 3-5 days and was ready to use after 11-15 days.

6.2.3.2 **Western blotting**

Osteoblast isolated from LC or KO mice were seeded in 6-well plate at the density of 150000cells/well in medium for 24 hours. The medium was replaced with phenol-red free DMEM for another 24-hour incubation. Basal expressions of ERs, including ER-α66, ER-α36, and GPER, in osteoblast isolated from LC or KO mice, were measured by western blotting. Procedures of western blotting were described in chapter 3.

6.2.3.3 **MTS assay**

Osteoblast isolated from LC or KO mice were seeded in 96-well plate at the density of 8000cells/well in medium for 24 hours. The medium was changed to phenol-red free DMA for 24-hour incubation before treatment with E_2(10^{-12}M, 10^{-10}M and 10^{-8}M), icariin (10^{-8}M, 10^{-7}M and 10^{-6}M) as well as EXD (0.1 μg/ml, 10 μg/ml and 100μg/ml) for 24 hours. MTS assay was performed, as illustrated in chapter 4.

6.2.3.4 **Statistical analysis**

Inter-group difference in LC and KO group *in vivo* study were determined by Student's T-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. *In vitro* study was analysed by one-way ANOVA, followed by Tukey's multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. graphs in this study were plotted by using GraphPad Prism Version 7.0.

6.3 Results

6.3.1 *In vivo* study

6.3.1.1 Genotyping

In figure 6.2, mice with homozygous wide-type (WT) ERα gene, with no loxP sequences inserted, generated a PCR product of 1200 bp. A floxed ERα gene, with the addition of the two loxP sequences, generated a 1280 bp. A heterozygous mouse in which one allele for ERα was floxed and the other allele was not $ERα^{fl/+}$ had PCR products of both sizes. Regarding Cre recombinase, the presence of 300 bp indicated the insertion of OC-Cre recombinase in the genome. Mice with homozygous floxed ERα and Cre recombinase were utilized as pOC-ERαKO (KO) mice while mice with no cre recombinase or homozygous WT ERα were utilized as littermate control (LC) mice.

6.3.1.2 Effect of pOC-ERαKO on bone properties in female mice

Results in figure 6.3 demonstrated that the knockout of ERα in osteoblasts significantly suppressed the femur length, but not tibia length in female mice (P<0.01 vs. LC group). In addition, the effects of pOC-ERαKO on bone mineral density (BMD) and microarchitectural properties at the proximal tibia, distal femur, and lumbar vertebra (L4) in mice were illustrated with their representative three-dimensional images and parameters obtained from micro-CT analysis in Figure 6.4. In the proximal tibia, knockout of ERα in osteoblast significantly suppressed connectivity density (Conn.D, 1/mm3), trabecular number (Tb.N, 1/mm), BMD, but increased trabecular separation (Tb.Sp, mm) in mice (P<0.05 vs. LC group). In distal femur, significant suppression on bone volume/tissue volume (BV/TV) and BMD (P<0.05 vs. LC group) and a notable increase in Tb.Sp (P<0.01 vs. LC group) were observed in the trabecular bone in pOC-ERαKO mice. As for the lumbar spine, pOC-ERαKO mice exhibited significant deterioration of BV/TV, trabecular thickness (Tb.Th, mm), and BMD (P<0.05 vs. LC group). Moreover, knockout of ERα in osteoblast remarkably reduced cortical area (Cr.Ar, mm^2), cortical thickness (Ct.Th,mm) and cortical area fraction (Ct.Ar/Tt.Ar) in midshaft of the femur of female mice (P<0.05 vs. LC group) (Figure 6.5). Similarly, significant decrease in the total cross-sectional area and marrow area were observed in the midshaft of tibia in pOC-ERαKO female mice (P<0.05 vs. LC group).

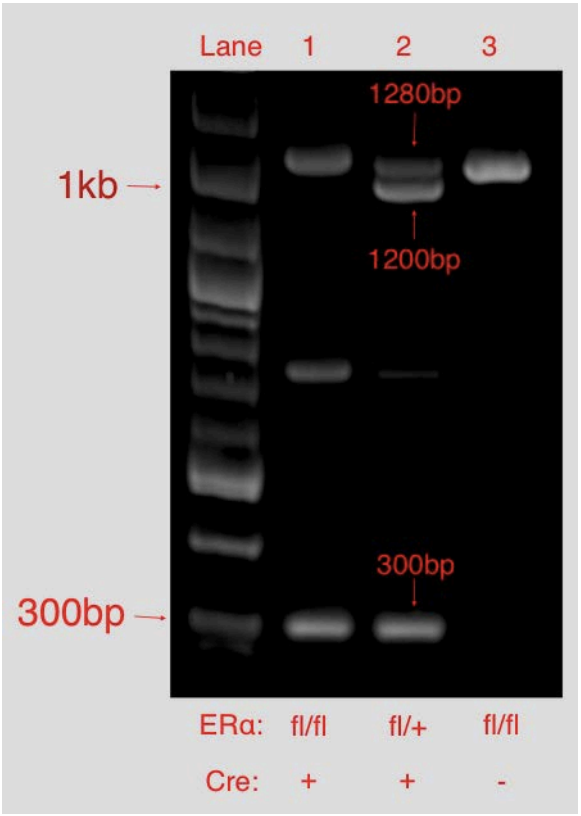


Figure 6.2 Genotyping of pOC-ERαKO mice.

Genomic DNA extracted from tail clips were analyzed by PCR. Homozygous floxed ERα$^{fl/fl}$ DNA generated a single product of 1280 bp. Heterozygous DNA ERα$^{fl/+}$ produced two PCR bands – 1200 and 1280 bp. Positive for the Cre transgene driven by the OCN promoter had a 300 bp product. Lane1: a single 1280 bp product was generated for floxed ERα and positive for Cre recombinase; Lane 2: a single 1280bp and a single 1200bp produced was generated for both floxed and non-floxed ERα and positive for Cre recombinase; Lane 3: a single 1280 bp product was generated and negative for Cre recombinase.

(a) (b)

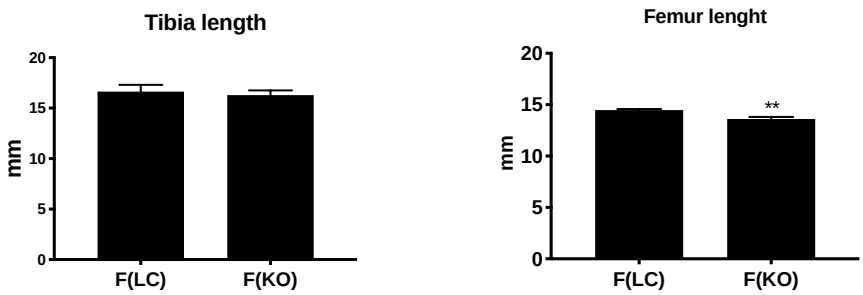


Figure 6.3 Effect of pOC-ERαKO on the length of proximal tibia (a), and distal femur (b) in female mice.

Left tibia and femur of littermate control (LC) and pOC-ERαKO mice (KO) were collected upon sacrifice. The length of tibia and femur of each mice were measured. Data were presented by mean ± SEM and analyzed by Student's t-tests. ** $P < 0.01$ vs. LC group. n=6-8.

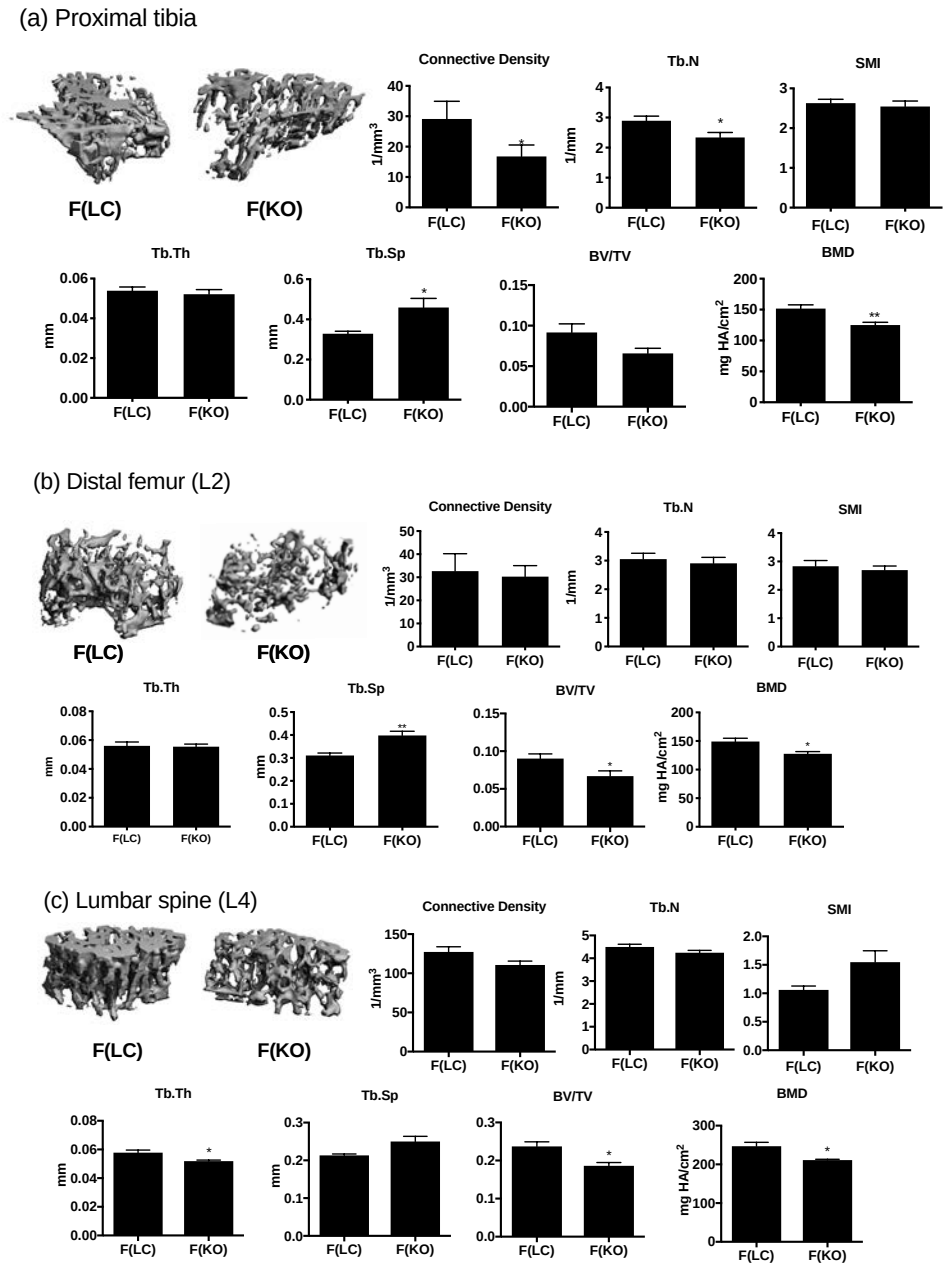
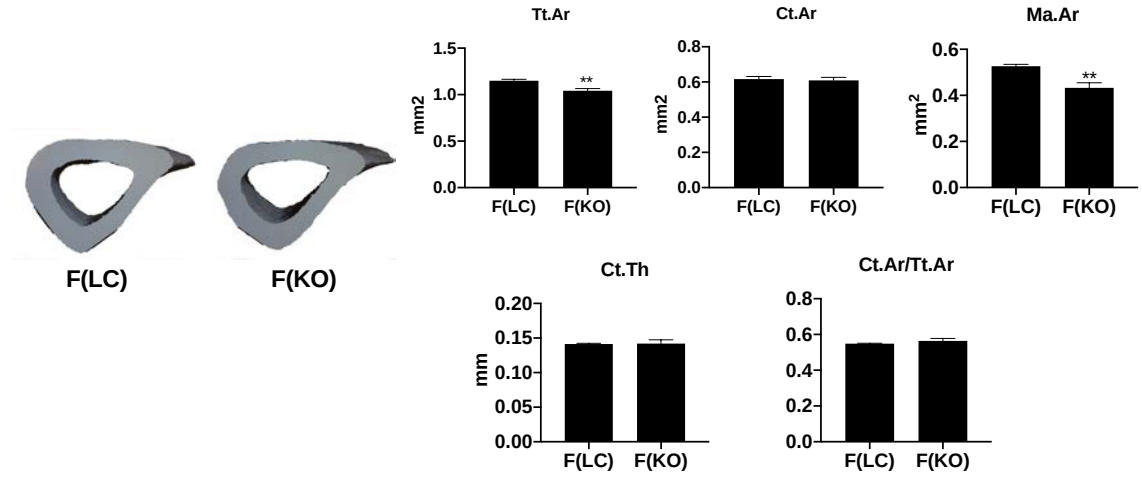


Figure 6.4 Effect of pOC-ERαKO on the bone microarchitecture of the trabecular bone in proximal tibia (a), distal femur (b) and lumbar vertebra L4 (c) in female mice.

Left tibia, femur, and lumbar vertebra of littermate control (LC) and pOC-ERαKO mice (KO) were collected upon sacrifice and scanned at high resolution by the micro-CT system (viva-CT40, Scanco Medical, Switzerland). A constant threshold of 375 was used to generate three-dimensional images for all samples. Bone microarchitecture parameters include bone volume/tissue volume (BV/TV), connectivity density (Conn.D), structural model index (SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). Data were presented by mean ± SEM and analyzed by Student's t-tests. * $P < 0.05$ vs. LC group. n=6-8.

(a) Proximal tibia



(b) Distal femur (L2)

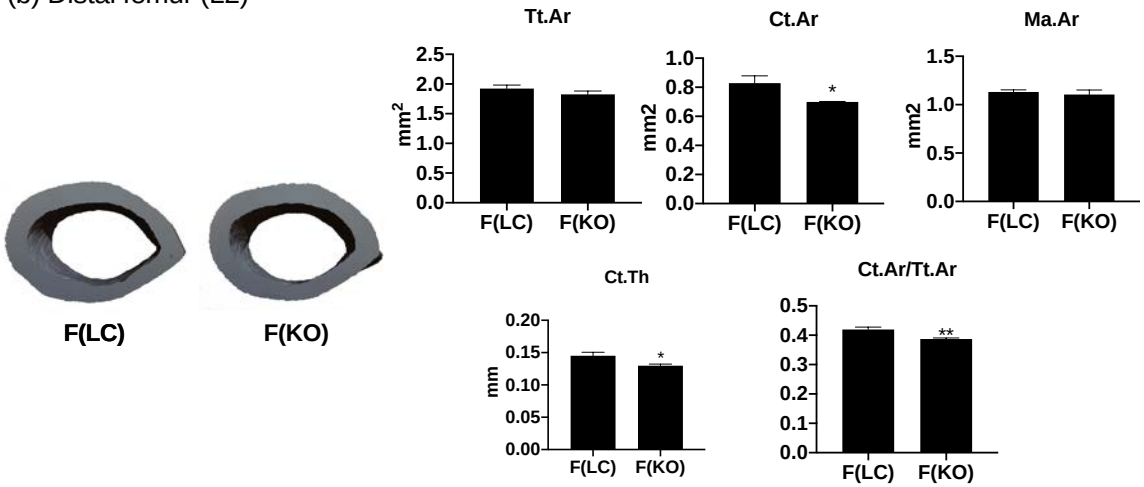


Figure 6.5 Effect of pOC-ERαKO on the bone microarchitecture of the cortical bone in proximal tibia (a), and distal femur (b) in female mice.

Left tibia and femur of littermate control (LC) and pOC-ERαKO mice (KO) were collected upon sacrifice and scanned at high resolution by the micro-CT system (viva-CT40, Scanco Medical, Switzerland). The bone at the midshaft of the femur and tibia were scanned using the same scanning condition as trabecular bone and 2D analysis was conducted on a single section to obtain total cross-sectional area (Tt.Ar, mm2), cortical area (Ct.Ar, mm2), marrow area (Ma.Ar, mm2), cortical thickness (Ct.Th, mm) and cortical area fraction (Ct.Ar/Tt.Ar). Data were presented by mean ± SEM and analyzed by Student's t-tests. * $P < 0.05$ vs. LC group. n=6-8.

6.3.1.3 Effect of pOC-ERαKO on mRNA expression of bone markers and GPER in tibia head

In figure 6.6, pOC-ERαKO dramatically reduced the mRNA expression of bone formation marker (OPG) by 0.3-fold in tibia heads (P<0.05 vs. LC group). Also, pOC-ERαKO tended to increase the mRNA expression of bone differentiation marker (ALP) and suppress the mRNA expression of bone resorption marker (RANKL) and GPER in tibia heads, but the changes did not reach statistical significance.

6.3.2 *Ex vivo* study

6.3.2.1 Effect of pOC-ERαKO on protein expression of ERs in osteoblasts

In figure 6.7, the basal protein expression of ER-α66 was the highest amongst ER-α66, ER-α36, and GPER in the LC group. Osteoblasts directly isolated from bone chips of pOC-ERαKO mice had a significant reduction of ER-α66 protein expression when compared to the LC group. Also, the expression of ER-α36 and GPER proteins were significantly increased by 2-fold and 3.5-fold, respectively, in response to osteoblasts-specific knockout of ER-α (P<0.05 vs. LC group).

6.3.2.2 Effect of pOC-ERαKO on icariin- or EXD- induced cell proliferation in osteoblasts

Results in figure 6.9 showed that E_2 (10^{-12}M, 10^{-10}M and 10^{-8}M), icariin (10^{-8}M, 10^{-7}M and 10^{-6}M) as well as EXD (0.1 μg/ml, 10 μg/ml and 100μg/ml) could remarkably (P<0.001 vs. LC control group) enhance the cell viability of osteoblast isolated from LC mice in 24 hours. Moreover, the basal cell viability of osteoblasts isolated from KO mice was comparatively (P<0.01 vs. LC control group) lower than that from the LC group. Also, E_2 (10^{-12}M, 10^{-10}M), but not 10^{-8}M, could obviously (P<0.001 vs. KO control group) enhanced the cell viability of osteoblast of KO mice. Three doses of icariin and EXD acted like E_2 to apparently induce (P<0.001 vs. KO control group) the cell viability of osteoblast of KO mice. Last, ERαKO in osteoblasts could solely lead to significant (P<0.001 vs. LC treatment group) suppression of E_2 (10^{-8}M)-induced cell viability of osteoblast when LC compared to treatment group.

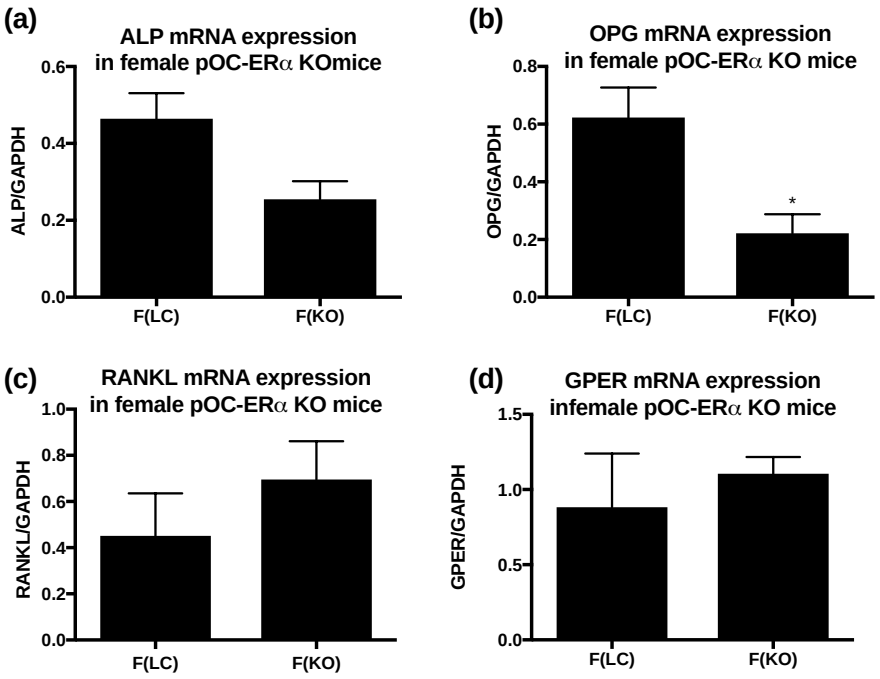


Figure 6.6 Effect of pOC-ERαKO on mRNA expression of bone markers and GPER in tibia head of female mice.

Tibia heads were isolated from mice upon sacrifice, and mRNA was obtained to analyse the expression of ALP (a), OPG (b), RANKL (c), and GPER (d) by real-time PCR. (a) The mRNA expression level was shown as the ratio of the target gene to GAPDH. Data were presented by mean ± SEM and analyzed by Student's t-test. n=3-6.

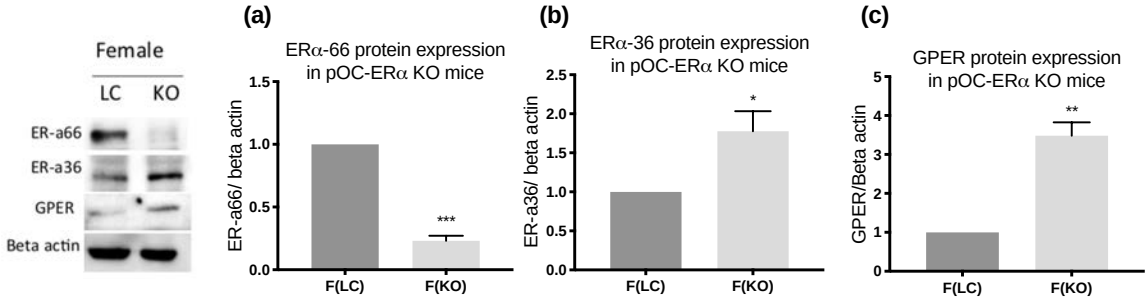


Figure 6.7 Effect of pOC-ERαKO on protein expression of ERs in primary osteoblast of female mice.

Proteins from osteoblast isolated from littermate control (LC) or pOC-ERαKO (KO) mice were extracted to analyse the expression of ER-α66(a), ER-α36(b), and GPER(c). The figures (d) are the representatives of immunoblot from three independent trials. The protein expression level was shown as the ratio of the target protein to β-actin. Data were presented by mean ± SEM and analyzed by Student's t-test. * P<0.05, ** P <0.01, *** P <0.001 vs littermate control group. n=3.

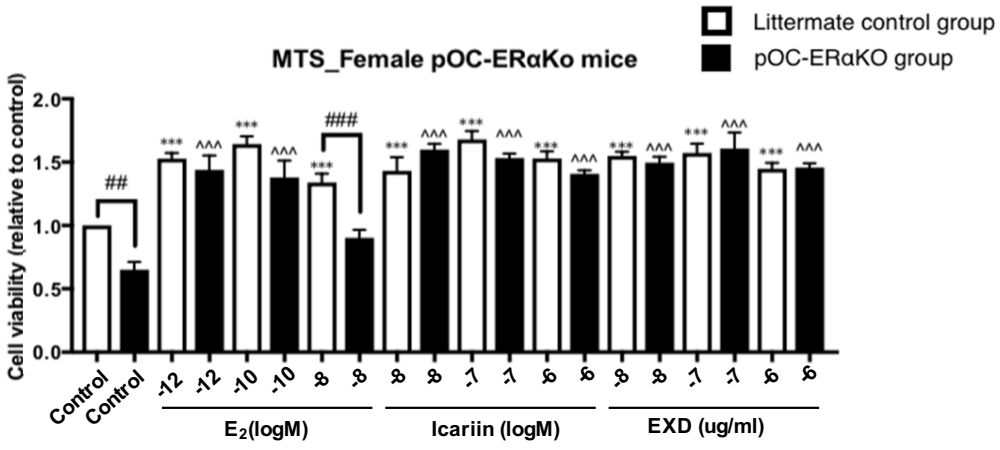


Figure 6.8 The effect of pOC-ERαKO on icariin- or EXD- induced cell viability in primary osteoblasts.

Primary osteoblasts isolated from littermate control (LC) or pOC-ERαKO (KO) mice were treated with E_2(10^{-12}M, 10^{-10}M and 10^{-8}M),icariin (10^{-8}M, 10^{-7}M and 10^{-6}M) as well as EXD (0.1 μg/ml, 10 μg/ml and 100μg/ml) for 24 hours. Cell viability was assessed by MTS assay. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. *** $P < 0.001$ vs. LC control group; ^^^ $P < 0.001$ vs. KO control group, ## $P < 0.01$, ### $P < 0.001$ vs. LC treatment group. n=3.

6.3.2.3 **Effect of pOC-ERαKO on icariin- or EXD- induced OPG/RANKL in osteoblasts**

In figure 6.7, 24-hour treatment of E_2 (10^{-12}-10^{-8}M), icariin (10^{-8}-10^{-6}M), or EXD (1μg/ml-100μg/ml) significantly induced the OPG/RANKL ratio in osteoblasts of LC mice by 1.5-, (P<0.001 vs. LC control group). On the other hand, the basal OPG/RNAKL in osteoblast was remarkably abolished by osteoblast-specific knockout of ER-α. ERαKO in osteoblasts abrogated E2-, icariin- and EXD- induced OPG/RANKL ratio in comparison with LC groups (P<0.001). There are significant difference of OPG/RANKL ratio between LC and KO group treated with E2, icariin and EXD (P<0.001 vs. LC control group).

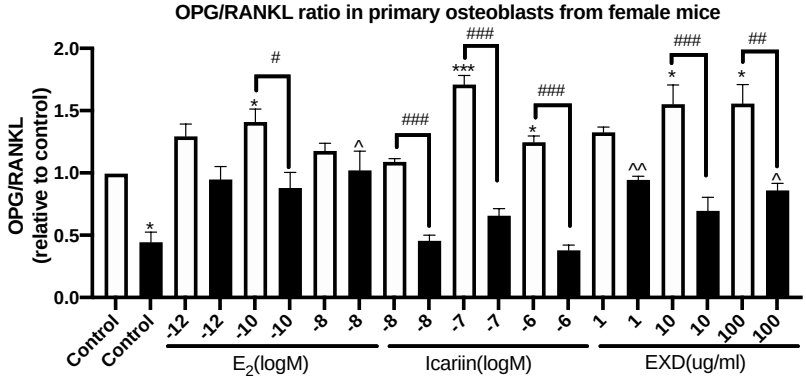


Figure 6.9 The effect of pOC-ERαKO on icariin- or EXD- induced OPG/RANKL ratio in primary osteoblasts.

Osteoblast isolated from littermate control (LC) or pOC-ERαKO (KO) mice were treated with vehicle, E_2 (10^{-12}-10^{-8}M), icariin (10^{-8}-10^{-6}M), or EXD (1μg/ml-100μg/ml) for 24 hours. mRNA expression of OPG and RANKL, were studied by western blotting. The figures are the representatives of three independent experiments. The expression level was shown as the ratio to GAPDH. Data were presented by mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. * P <0.05 vs. LC control group; ^ P <0.05, ^^ P <0.01 vs. KO control group, # P < 0.05, ## P <0.01, ### P <0.001 vs. LC treatment group. n=3.

6.4 Discussion

In this chapter, we have demonstrated that ER-α66 is essential for basal bone formation using osteoblast-specific ERα knockout mice. We also showed differences in the ER-α66-dependency of the actions of E_2, icariin and EXD on bone formation. Interestingly, the increase in protein expression of novel ERs were observed in osteoblast of pOC-ERαKO mice, which might compensate for the loss of ER-α66 in mediating rapid estrogenic signaling and cell proliferation induced by icariin .

ERα in osteoblast is essential for basal bone formation

Bone deterioration was observed in both cortical and trabecular bone of the proximal tibia and distal femur as well as trabecular bone of lumbar spine of pOC-ERαKO mice. It indicated that ERα played an essential role in bone formation. Our results were in line with the previous study by Dr. Marjolein van der Meulen at Cornell University that osteocalcin-drive osteoblast-specific ERαKO could reduce the cancellous and cortical bone mass of proximal tibia, L5 vertebra in 12-week-old female mice (Melville et al., 2014; Melville et al., 2015). Rooney's group also revealed that the presence of ERα in osteoblast is curial for trabecular bone volume in the tibia, femur as well as vertebra and cortical bone volume using bacteriophage-derived osteoblast-specific ERαKO mice (Rooney & van der Meulen, 2017). Furthermore, our group first determined the mRNA expression of different bone markers in tibia head of pOC-ERαKO mice. The results indicated that the mRNA expression of bone formation marker (OPG) was reduced after knockout of ERα in osteoblast. In other words, the bone worsening in mice might be due to the reduction of OPG expression. OPG is a RANKL receptor-like molecule, which is tightly regulated in osteoblast linage cells by estrogen via binding to ER-α. OPG could inhibit osteoclastogenesis via the prevention of RANKL/RANK formation(Yasuda et al., 1998). OPG-deficient mice were found to have early onset of osteoporosis (Bucay et al., 1998). Thus, the knockout of ERα in osteoblast might abolish endogenous E_2 induced mRNA expression of OPG, which further increase the rate of osteoclastogenesis. However, our results suggested that the lack of ER-α in osteoblast may not affect cell differentiation and bone resorption in tibia head.

The loss of ERα in osteoblast could lead to the increase in ER-α36 and GPER expression

As it was not clear if the deletion of exon 3 on Esr1 in pOC-ERαKO mice altered the expression of other ERs, the basal mRNA and protein expression of ER-α66, ER-α36, and GPER in osteoblast from LC and KO mice were compared. The basal expression of ER-α66 was found

to be dominant in osteoblast when compared to ER-α36 and GPER. Moreover, pOC-ERαKO mice have a nearly undetectable level of protein expression of ER-α66, but higher protein expression of ER-α36 and GPER. The increase in ER-α36 and GPER expression could be due to the relief of the negative feedback on their expression due to the lack of ER-α66 in pOC-ERαKO mice.

ER-α36 is believed to be transcribed from a previously undetermined promoter located in intron 1 of ESR1 (Soltysik & Czekaj, 2015). Intact intron 1 pf ESR1 in pOC-ERαKO mice might account for the expression of ER-α36. Also, the differential regulations of ER-α66 and ER-α36 are also reported by others in which the addition of E_2 could reduce ER-α36 expression while increase ER-α66 expression in osteoblasts harvested from postmenopausal women (Xie et al., 2011). ICI 182,780, an estrogen receptor-specific antagonist, could suppress the protein expression of ER-α66 while promoting ER-α36 expression in breast tissues (Z. Y. Wang & Yin, 2015). Moreover, knockout of transcriptional factor Wilms' tumor suppressor gene (WT1) was found to reciprocally regulate ER-α36 and ER-α66 expression in human breast MCF-7 cells. WT1, a dual transcription regulator, was also reported to activate the promoter activity of ER-α66 while suppressing ER-α36 promoter activity in HEK293 cells (L. Kang et al., 2011). Thus, a relief of the negative feedback of ER-α36 protein expression in mice might happen when ER-α66 protein expression is reduced.

GPER protein expression, which behaved like ER-α36, was shown to be oppositely regulated in response to a lack of ER-α66 in mice osteoblast. These results are consistent with other studies that introducing GPER could induce endogenous ER-α36 in GPER non-expressing HEK293 cells, while knockdown of GPER could lead to downregulation of ER-α36 in breast cancer SK-BR-3 cells (Lianguo Kang et al., 2010). Thus, the induction of GPER protein expression in mice might be due to relief of negative feedback when ER-α66 protein expression is reduced. Interestingly, no significant change of mRNA expression of GPER was observed in the tibia head of KO mice when compared to the LC group. Growth plate in tibia head consists of a board range of bone cells and adipocytes derived during adipogenesis and osteoblastogenesis of BMSCs. GPER was reported to have differential roles in regulating both adipogenesis and osteoblastogenesis. Studies found that activation of GPER could promote osteoblastic differentiation in MC3T3-E1 cells and inhibit adipogenesis in murine pre-osteoblast 3T3-L1 Cells. Thus, the mRNA expression of GPER in tibia head might not reflect its response to bone formation, but suggest its action in both osteblasts and adipocytes. In order to have a better understand of the role of GPER in bone formation in pOC-ERαKO mice, the mRNA expression of GPER in osteoblats should be further studied.

ER-α66 and ER-α36 might respond to different E_2 level

Postmenopausal osteoporosis is mainly caused by the decline of serum estrogen level, which induce a local imbalance between bone resorption and formation mediated by ER via estrogen signaling. Therefore, we have studied the influence of ER-α66 in cell viability and rapid estrogen signaling in osteoblasts from pOC-ERαKO mice. Our results showed that normal physiological E_2 level (10^{-8}M) and postmenopausal low-level E_2 (10^{-12}M -10^{-10}M) could promote cell viability of osteoblasts derived from normal mice in 24 hours. In contrast, postmenopausal E_2 level (10^{-12}M -10^{-10}M), but not normal physiological E_2 level (10^{-8}M), induced cell viability of osteoblasts derived from pOC-ERαKO mice. The results indicate that ER-α66 is indispensable for the growth of osteoblasts at normal physiological E_2 level, and seems to have less effect at postmenopausal E_2 level. In other words, a low-level of E_2 might recruit distinct estrogen responders in osteoblastic cells when compared to the normal level of E_2. Previously, Wang proposed that ER-α36, but not ER-α66, might participate in cell proliferation of osteoblast isolated from normal postmenopausal women. In that study, normal postmenopausal women express high levels of endogenous ER-α36 and respond to 10 pM E_2 (postmenopausal level of E_2). The same concentration of E_2 produced significant mitogenic and anti-apoptotic effects on osteoblasts which could be abloshed by ER-α36 knockdown in osteoblasts (Xie et al., 2011). Therefore, it is anticipated that the high protein expression of ER-α36 in osteoblasts of KO mice may account for the stimulation of osteoblastic cell viability at low-level E_2. Further study examining the protein expression ER-α36 upon low-level E_2 in osteoblasts is required to verify the correlations between ER-α36 expression and low-level E_2 -induced cell viability or signaling induction. Collectively, ER-α36 and ER-α66 might be responsive to different level of E_2 level in which ER-α36 might play an important role in regulating bone metabolism at the low E_2 levels of postmenopausal women.

Only limited studies have explored the role of GPER in bone health of postmenopausal women. A recent preclinical study using OVX mice showed that the administration of GPER-specific agonist (G1) did not alter OVX-induced reduction of BMD in mice (Iravani et al., 2019), suggesting that GPER might not respond to low concentration of E_2 in bone. However, in chapter 3, we have demonstrated that GPER could regulate the expression of ER-α36 in osteoblastic cells. Also, GPER was reported to physically interact with ER-α36 on the cell membrane as a coregulator in mediating anti-inflammatory action of E_2 in human primary monocytes (Pelekanou et al., 2016). Thus, it is anticipated that the increase of GPER expression

in osteoblast from pOC-ERαKO mice might account for the regulation of ER-α36 instead of playing a direct role in bone remodelling.

These results provided a new insight that different levels of E_2 might recruit distinct ERs for mediating estrogenic event. Follow-up study on the effect of low-level of E_2- induced ERK phosphorylation in osteoblast of KO mice with ER-α36 overexpression is needed in order to investigate if ER-α36 is the functional ER in postmenopausal women. Also, a study about GPER-dependent regulation of ER-α36 expression in osteoblast of KO mice under a low-level of E_2 will be needed for the understanding of the role of GPER in postmenopausal bone remodelling.

ER-α36 and GPER might participate in ER-α66-independent bone protection of icariin and EXD

ER-α36 and GPER were reported to mediate estrogen induction in both ER-α66-positive and ER-α66-negative cells (Gaudet et al., 2015; Soltysik & Czekaj, 2015). Thus, we have utilized osteoblast from pOC-ERαKO mice to study the distinct roles of ER-α36 and GPER in mediating ER-α66-independent icariin-or EXD-induced bone protection. Our results suggested that icariin and EXD might stimulate osteoblastic growth in an ER-α66-independent manner. Lack of ER-α66 in osteoblasts did not alter icariin-or EXD-induced cell proliferation in osteoblasts. The results indicated that other receptors in the cells, like ER-α36 and GPER, might trigger responses to these treatments. Further study using knockdown of ER-α36 and GPER is needed to verify if icariin and EXD could activate osteoblastic cell proliferation.

ER-α66 is important in regulating estrogen sensitive OPG/RANKL ratio upon EXD and icariin treatment in osteoblast derived from ER-α66-knockout mice

pOC-ERαKO could supressed the OPG/RANKL ratio without any treatment and when the cells treated with E_2 , icariin and EXD. The results signified that EXD and icariin might require ER-α, especially ER-α66, in altering the gene expression of OPG and RANKL. It has been proved that ER-α66 could exert ERE-dependent genomic estrogen signaling (Lianguo Kang et al., 2010)while ER-36(Omarjee et al., 2017 and GPER could only trigger rapid estrogen signalling and did not activate ERE. Therefore, it is anticipated that icariin and EXD could exert ER-dependent effect in bone.

Collectively, we have demonstrated that ER-α66 is vital in basal bone remodelling in mice (figure 6.10). Also, we elucidated the different actions of ER-α66 and ER-α36 in osteoblast. ER-α66 perhaps worked dominantly in the normal physiological E_2 level while ER-α36 is highly sensitive to E_2 and works dominantly in the postmenopausal E_2 level in osteoblast. Also, the bone protection of EXD and icariin is ER-α66-dependent in term of regulation of E_2-

sensitive gene. Further study is required to confirm if ER-α36 or GPER mediates the icariin- or EXD activated cell proliferation in the absence of ER-α66 in osteoblast. Moreover, it is still unclear if GPER participates in the bone protection of icariin and EXD in osteoblast via regulating ER-α36 expression. Further studies are desired to study the role of GPER in icariin or EXD action in bone in pOC-ERαKO mice.

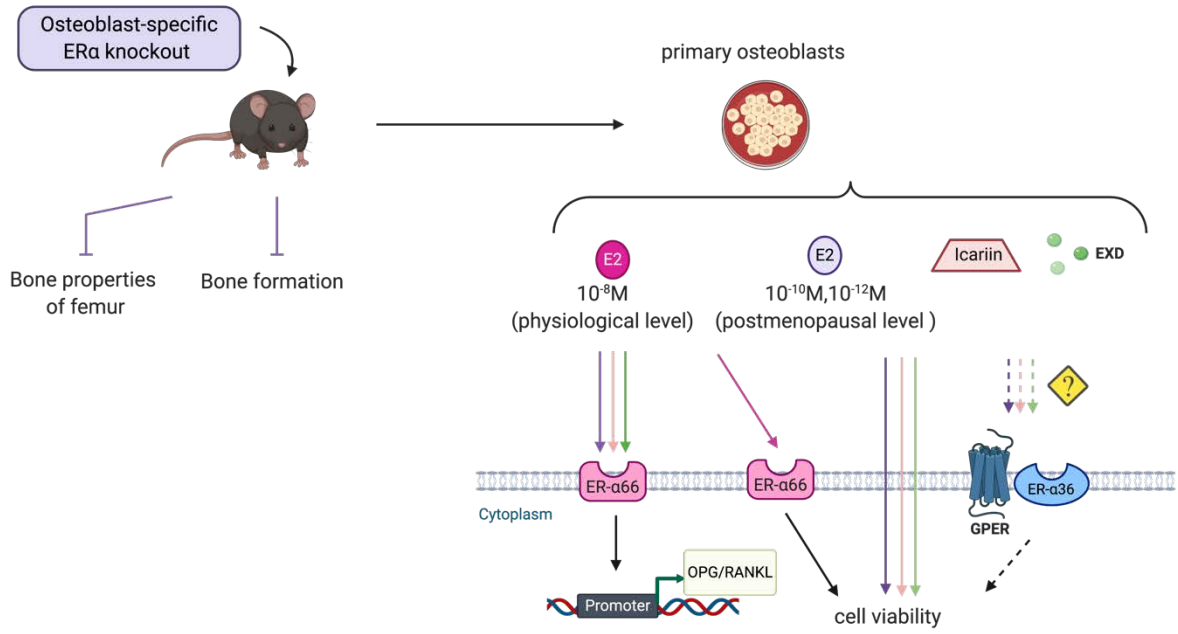


Figure 6.10 A brief summary illustrating the dependency of ERs in bone protection of E_2 ,icariin and EXD.

pOC-ERαKO mice showed to have weaker bone properties in both trabecular and cortical bone of femur and tibia. The bone deterioration of tibia might be caused by reduction of OPG mRNA expression in tibia head. pOC-ERαKO mice also had poor bone condition in lumbar spine. As for *ex vivo* study, normal physiological level E2 (10^{-8}M) required ER-α66 for cell proliferation while postmenopausal level E2 (10^{-12}M and 10^{-10}M), EXD and icariin could bypass ERα for inducing ER-α66-independent cell proliferation of mice primary osteoblasts. Also, EXD, but not icariin, required ER-α66 for phosphorylation of ERK in mice primary osteoblasts. Further study is needed to investigate if icariin recruits other ERs, like ER-α36 or GPER, to trigger ERK pathway because knockout of ERα66 in osteoblast increased the expression of these two extranuclear ERs.

Chapter 7. Discussion and conclusion

7.1 Discussion

With the increased concern of severe side effects associated with the existing drug therapy, people started to look for alternative approaches, such as phytoestrogen and Traditional Chinese Medicine (TCM) for treatment of postmenopausal osteoporosis as they are naturally occurring plant-derived remedies with safety profiles that might be accessible for the treatment of hormone-related diseases (Che et al., 2016). Icariin is a phytoestrogen that could activate estrogen receptors to promote bone formation and suppress bone resorption. Our group, previously, indicated that icariin acted like a pathway-specific Selective Estrogen Receptor Modulator (SERM) exerting bone protective effects via membrane initiated, extranuclear, ligand-independent estrogen signaling pathway. However, the therapeutic target of rapid estrogen pathway mediated by icariin is still unknown as it does not bind to classical estrogen receptor alpha (ER-α or now called ER-α66). On the other hand, icariin-containing TCM, Er-xian decoction (EXD) have been clinically prescribed to manage postmenopausal osteoporosis. Our group has shown that EXD could promote bone formation via both estrogen-responsive element (ERE)-dependent genomic and rapid estrogenic pathways (K. C. Wong et al., 2014). The estrogen signaling and effectors regulated by EXD in bone formation are far from clear due to the complexity and interaction of the active components in EXD. In the present study, we explored the involvement of two novel membrane estrogen receptors, estrogen receptor alpha 36 (ER-α36) and G-protein coupled estrogen receptor (GPER) in rapid estrogenic events activated by icariin or EXD in bone.

ER-α36 is a new isoform of ER-α66, synthesized by alternative splicing of ESR1 and transcribed from a previously unidentified half-ERE-containing promoter in the first intron of ER-α66 (Z. Wang et al., 2005). It could either activate or deactivate the rapid estrogenic MAPK/ERK pathway as ERK2 could directly bind to the domain D of ER-α36, which in turn is recognized by its activators, substrate, and regulators for both phosphorylation and dephosphorylation (Omarjee et al., 2017).In addition, GPER could trigger a variety of signaling cascades, and subsequently undergo receptor endocytosis to avoid excessive signaling, a process referred to as receptor desensitization in response to E_2 (L. Wang et al., 2009). Formerly, the expressions of GPER and ER-α36 were associated with bone growth. The knockdown of GPER in mice was discovered to increase bone mass, femur size, cortical thickness, and mineralization (Ford et al., 2011). The upregulation of ER-α36 was also found to be associated with postmenopausal osteoporosis occurrence in the clinical study (Xie et al., 2011). These studies suggest that extranuclear ER-α36 and GPER, beside classical ER-α66,

could be suitable candidates for mediating rapid estrogenic signaling induced by icariin and EXD during osteoblastogenesis.

Before studying the roles of ER-α36 and GPER in bone formation, it is of utmost importance to first confirm if bone tissues, especially osteoblasts, could express these two novel receptors. In chapters 3 and 5, we utilized tibia head from ovariectomized (OVX) Sprague Dawley (SD) rats treated with 12-week icariin or EXD *in vivo*, human osteoblast-like MG-63 cells and murine preosteoblast MC3T3-E1 cells *in vitro*. Data showed that ER-α36 and GPER were expressed in rat tibia heads and osteoblasts at basal level. In other words, ER-α66 is not the sole ER-α in bone. Next, we analyzed how these ERs respond to E_2, icariin or EXD in bone. *In vivo* results illustrated that the protein expressions of ER-α66, ER-α36, and GPER were significantly increased in tibia heads of OVX rats, which could be reversed by 12-week treatment of E_2, icariin or EXD. *In vitro* studies suggested that E_2, icariin or EXD could boost the expression of ER-α66, but reduce the expression of ER-α36 and GPER in both MC3T3-E1 cells and MG-63 cells. Despite the different responses of ERs expressions between *in vivo* and *in vitro*, it implies that E_2, icariin or EXD could regulate all three estrogen receptors. Tibia heads consist of bone marrow, osteoclast, osteoblasts, adipocytes, and chondrocytes. Thus, the protein expression of these ERs might reflect their physiological roles in the growth plate of epiphysis. More precisely, *in vitro* studies could contemplate the response of these ERs in osteoblast, directly involved in bone formation. After all, it is suggested that E2, icariin or EXD could regulate the protein expression of ER-α66, ER-α36, and GPER in bone. Also, the protein expressions of ER-α36 and GPER were differentially regulated when compared to ER-α66 in osteoblasts upon these treatments.

Subsequently, we examined the interactive regulation of protein expression amongst these ERs. Earlier on, researchers discovered that ER-α36 is synthesized by alternative splicing of ESR1 and transcribed from a previously unidentified half-ERE-containing promoter in the first intron of ER-α66 (Z. Wang et al., 2005). Also, few studies suggested that ER-α36 is likely a downstream target of GPER as the knockdown of GPER could reduce both GPER and ER-α36 in human neuroblastoma SHSY-5Y cells (Han et al., 2015). These implied a crosstalk amongst ERs on their expression. It is of interest to study if the differential regulations of ERs in response to E_2, icariin or EXD in osteoblast result from ERs' crosstalk. In the present study, ER-α66-negative human embryonic kidney 293 (HEK293) cells were knocked in with ER-α66, overexpressed with ER-α36 or GPER. It was shown that knockin of ER-α66 in HEK293 cells could promote ER-α66 protein expression without altering the expression of GPER and ER-α36. On the contrary, overexpression of GPER increased both GPER and ER-α36 expression

and stimulated ER-α36 promoter activity expression while overexpression of ER-α36 did not affect both GPER and ER-α66 expressions. On the other hand, the treatments of E_2 and icariin could suppress both GPER and ER-α36 expression in HEK293 cells without altering ER-α66. These results not only further confirmed the differential regulation of ER-α66 expression compared to GPER and ER-α36, but also suggested that the regulation of ER-α36 expression by E_2 or icariin is GPER-dependent and ER-α66-independent.

GPER and ER-α36 expressions were reported to correlate with physiological disorders. However, their roles in osteoporosis and bone remodelling remain unclear. Thus, after studying the responsiveness of ERs toward E_2, icariin, and EXD in bone, we tried to explain the physiological meaning of GPER and ER-α36 expressions in the rapid estrogenic effect of icariin or EXD in bone. In chapter 4, MC3T3-E1 cells and MG-63 cells, which basally express a high level of ER-α66 and GPER and low level of ER-α36, were transfected with ER-α36 expression vector or GPER siRNA or pre-treated with G15, a GPER specific antagonist. GPER knockdown could further enhance icariin-induced, but not E_2-induced, cell viability, OPG mRNA expression in both cells as well as OCN mRNA expression in MG-63 cells. Moreover, blocking GPER further increased the phosphorylation of Akt, ERK, and ER-α (Ser167) in both cells upon E_2 treatment for 10-minutes and boosted icariin-induced phosphorylation of ERK and ER-α (Ser167) in MC3T3-E1 cells. Similarly, GPER knockdown caused reinforcement of the phosphorylation of Akt in both cells upon treatment with icariin for 10-minute. These not only indicated that icariin and E_2 might have a different mechanism in modulating bone formation, but also GPER is likely a negative regulator in icariin's bone protection and activation of rapid estrogenic signaling. As for ER-α36, ER-α36 overexpression could significantly reduce icariin-induced expression of ALP and OCN mRNA in MC3T3-E1 cells, as well as the cell viability, mRNA expression of OPG and OPG/RANKL ratio in MG-63 cells. Moreover, overexpression of ER-α36 inhibited the rapid estrogenic signaling, including the phosphorylation of Akt and ER-α (Ser167) in MC3T3-E1 cells and ERK and ER-α(Ser167) in MG-63 cells upon 10-minute icariin treatment. In contrast, ER-α36 overexpression did not alter the phosphorylation of measured signaling molecules upon E_2 treatment in both cells. These results suggest that ER-α36 negates the actions of icariin in bone and that the actions of icariin are different from E_2 in osteoblastic cells.

GPER and ER-α36 are two membrane ERs that activate rapid estrogen signaling after cellular translocation. Also, GPER was reported to physically interact with ER-α36 on the cell membrane as a coregulator mediating anti-inflammatory action of E_2 in human primary monocytes (Pelekanou et al., 2016). Besides, ER-α36/ER-α66 heterodimer seems to prevent

the binding of ER-α66 to transcriptional factor in the nucleus by retaining ER-α66 in the cytoplasm (Z. Y. Wang & Yin, 2015). It is worthwhile to study if the rapid estrogenic bone protection of E_2 or icariin involves the crosstalk amongst ERs. Hence, we studied the cellular translocation of ERs. Immunofluorescence staining confirmed that 10-minute icariin and E_2 treatment in MC3T3-E1 cells and rat bone marrow stromal cells (rBMSCs) reduced the translocation of GPER and ER-α36 from cytoplasm toward the edge, but lead to translocation of ER-α66 from cytoplasm toward the nucleus. It is well known that ER-α66 could activate both rapid and genomic estrogen signaling in bone by the formation of ER dimers, followed by translocation to the nucleus in bone (Vrtačnik et al., 2014). Therefore, the present results might imply icariin and E_2 activate ER-α66-mediated rapid estrogen signaling, at the same time, inhibiting GPER and ER-α36 signaling activation in osteoblast. In particular, we investigated the formation of signaling complexes in the estrogen singling pathway. Immunoprecipitation assay suggested that icariin could reduce the formation of ER-α66/ER-α36 and ER-α66/c-src complexes but increase ER-α66/shc formation in rBMSCs. Only icariin could suppress the formation of ER-α66/c-src in rBMSCs upon treatment for 10 minutes. c-src, an upstream signaling molecule in PI3K/AKT or MAPK/PI3K pathway (Franke, 2008), has been reported to be a negative regulator in osteoblastogenesis in which knockdown of c-src was previously shown to enhance osteoblastic cell proliferation, differentiation and bone formation *in vivo* (Marzia et al., 2000). Suppressing ER-α66/ER-α36 heterodimer and ER-α66/c-src complexes in osteoblasts upon icariin treatment might prevent ER-α36 from interacting with c-src. Taken together, icariin might exert bone protection by reducing ER-α66/ER-α36 and ER-α66/c-src complexes which allow the translocation of ER-α66 to the nucleus for genomic and rapid estrogen signaling.

As for the EXD study, we again corroborated the tissue-specific and estrogen-like bone protective effect of long-term EXD treatment (1.6g/kg body weight/day) in three-month-old female OVX SD rats, and such effect might be due to hormone regulation. Tibia heads of OVX rats showed to have higher expressions of ER-α66, ER-α36, and GPER, which could be reversed by the administration of E_2 or EXD. *In vivo* study showed that E_2 and EXD treatment could suppress ER-α36 and GPER protein expressions in both MC3T3-E1 cells and MG-63 cells. Such observation is in agreement with a former study in which the addition of E_2 could reduce ER-α36 expression while increase ER-α66 expression in osteoblasts harvested from postmenopausal women (Xie et al., 2011). Then, overexpression of ER-α36 could abolish the effects of E_2 or EXD on cell viability and mRNA OPG/RANKL ratio in both cells. ER-α36 seems to be a suppressor in the Akt pathway and phosphorylation of ER-α (Ser118) and

(Ser167) in MC3T3-E1 cells upon 10-minute EXD treatment. Likewise, the ERK pathway and phosphorylation of ER-α (Ser118) and (Ser167) in ER-α36-overexpressed MG-63 cells were suppressed upon 10-minute treatment with EXD or E_2. Taken together, EXD and E_2 treatment appeared to downregulate ER-α36 expression preventing the inhibitory effects of ER-α36 on ER-α66-induced cell viability, bone remodelling, and rapid estrogenic signaling in osteoblasts. Regarding GPER, blocking GPER by G15 abolished its inhibitory effects on EXD-induced mRNA expression of OCN, OPG as well as OPG/RANKL ratio, and EXD- and E_2-induced OPG mRNA expression in both cells. Furthermore, GPER knockdown could further enhance EXD-induced cell viability and mRNA expression of OPG in both cells. It indicates that GPER involved in the bone protective effect of EXD ostensibly as a negative regulator on cell viability and bone formation. Next, the phosphorylation of Akt, ERK , ER-α (Ser118), and (Ser167) induced by EXD or E_2 were enhanced when GPER is being blocked or knockdown. It indicates that GPER participates as an inhibitory role in Akt and ERK pathway as well as ER-α66 phosphorylation in osteoblastic cells in response to EXD.

Interestingly, a study revealed that ER-α36 could activate MAPK/ERK signaling pathway at a low concentration of E_2 (10^{-12}M) in ER-α36-overexpressed osteoblast form Chinese postmenopausal women and the expression of ER-α36 positively correlates with BMD and negatively with bone turnover markers, collagen type 1 cross-linked N-telopeptide (P1NP) and OCN in Chinese postmenopausal women (Xie et al., 2011). Moreover, ER-α36 is reported to be involved in icaritin-induced growth inhibition of triple-negative breast cancer cells via activation of MAPK/ERK (X. Wang et al., 2017). It could trigger ERK phosphorylation in ER-α36-positive HEK293 cells (Z. Wang et al., 2006), ER-α66-negative breast SK-BR-3 cells (Pelekanou et al., 2012), as well as ER-α66 knockdown endometrial cancer Hec1A cells (Lin et al., 2010). Although these results advocated a contradictory effect of ER-α36 on ERK activation compared to the present study, it is worth to note that those cell models in others' studies express a low level of or even no ER-α66, while osteoblasts utilized in the present study are ER-α66-positive cell expressing detectable ER-α66. These findings suggested that ER-α36 seems to mediate estrogen induction in both ER-α66-positive and ER-α66-negative cells. Indeed, these findings provide an insight that the action of ER-α36 in estrogen-related events might be sensitive to E_2 level and depend on ER-α66 expression.

Due to complicated crosstalk among ERs, estrogen induction of rapid responses performed in intact cells does not distinguish between ER-α isoforms. Thus, we have employed female osteoblast-specific ER-α knockout (pOC-ERαKO) mice to study the ER-α66-dependency and

unique roles of different ERs in bone remodelling. pOC-ERαKO mice were generated by breeding mice with exon 3 of the DNA binding domain of the ERα gene (Esr1) flanked by loxP sequences, $ERα^{fl/fl}$, to mice containing a transgene encoding Cre recombinase driven by the human osteocalcin promoter, OC-Cre (Melville et al., 2014). Phenotypically, data in chapter 6 indicated that ERα knockout in osteoblast could lead to bone deterioration in both cortical and trabecular bone of the proximal tibia and distal femur, trabecular bone of lumbar spine as well as reduction of femur length of pOC-ERαKO mice. At transcriptional level, ERα knockout in osteoblast significantly reduce the mRNA expression of bone formation marker (OPG) and tended to reduce the mRNA expression of bone differentiation marker (ALP) without reaching statistical significance in tibia head of pOC-ERαKO mice as compared those of control group. These suggested ERα in osteoblast played a vital role in basal bone formation in mice. The knockout of exon 3 in ERα, DNA binding domain, could suppress the expression of ERE-containing bone markers, thereby worsening bone condition in pOC-ERαKO mice.

The involvement of ER-α36 and GPER in the effects on bone remodelling induced by E_2, icariin, and EXD was also examined in primary osteoblast isolated from pOC-ERαKO mice. Knockout of ER-α could promote the basal expression of ER-α36 and GPER in osteoblasts isolated from pOC-ERαKO mice as compared to those isolated from control group. It indicated that the relief of the inhibitory effects on ER-α36 and GPER expression due to the absence of ER-α66 in pOC-ERαKO mice. Moreover, we also demonstrated only postmenopausal E_2 level (10^{-12}M -10^{-10}M) could promote cell viability of primary osteoblasts from pOC-ERαKO mice; while both postmenopausal E_2 level and normal physiological E_2 level (10^{-8}M) increased cell viability in primary osteoblasts from control mice. These denoted that ER-α is indispensable for the growth of osteoblasts at the normal physiological E_2 level, and seems to have less effect at the postmenopausal E_2 level. In other words, distinct estrogen responders, such as ER-α36 or GPER, might be recruited by a low-level of E_2 in osteoblasts during bone formation when compared to those recruited by normal level of E_2.

As for icariin and EXD, lack of ER-α66 in osteoblasts did not alter icariin-or EXD-induced cell proliferation in mice primary osteoblasts. It manifested the ER-α66-independence of bone formation mediated by icariin and EXD, whereas, other receptors in the cells, like ER-α36 and GPER, might maintain bone formation upon these treatments. Besides, pOC-ERαKO further enhanced icariin-induced, but suppress EXD-induced phosphorylation of ERK in 10 minutes in mice primary osteoblast compared to control. It signified that EXD and icariin might activate rapid estrogen signaling in different ways. The activation of ERK by icariin in osteoblast from KO mice could be related to the high expression level of ER-α36. Structurally, ERK2 was

reported to directly bind to the domain D of ER-α36, which could be recognized by its activators, substrate, and regulators for both phosphorylation and dephosphorylation. Such a structural finding suggests that ER-α36 could either activate or deactivate the MAPK/ERK pathway (Omarjee et al., 2017). ER-α36 also mediates phosphorylation of ERK in low ER-α66/ER-α36 ratio osteoblast isolated from Chinese postmenopausal women (Xie et al., 2011). The activation of MAPK/ERK pathway via ER-α36, in the absence of ER-α66, was found in HEK293 cells treated with antiestrogen, tamoxifen and ICI 182,780 (Lianguo Kang et al., 2010). Thus, ER-α36 seemed to enhance the icariin-mediated ERK pathway in ER-α-negative osteoblasts.

The present study showed that icariin and EXD have similarities and differences in terms of recruiting ER-α36 and GPER in the rapid estrogenic protection effect. EXD and icariin might promote cell viability, OPG mRNA expression and phosphorylation of Akt which require ER-α36 and GPER as an negative regulator. This similarities between them could be explained by the content of icariin in EXD. Our HPLC results (Appendix 3) showed that 1.605mg icariin was presented in 1g EXD. The dose of EXD (100ug.ml) used in the present *in vitro* study contains 0.1605ug/ml icariin. It is equivalent to 2 $x10^{-7}$M which is comparable to the dose of icariin used in the present *in vitro* study. As for the differences between icariin and EXD, ER-α36 and GPER were found to be involved more in ER-α66-mediated bone protection upon EXD treatment than upon icariin treatment regarding the activation of phosphorylation of ERK, ER-α(ser167) and ER-α(ser118). This discrepancy might be due to the presence of other flavonoids in EXD, including Icariside II, Epimedin B and Sagittatoside A which were proven to exert bone protection via the action of ER-α or the activation of rapid estrogen signaling in UMR106 cells (Xiao et al., 2014). Further study should examine the roles of novel ERs in mediating the actions of these flavonoids.

Taken together, both ER-α36 and GPER see to be the negative regulators in ER-α66-dependent rapid estrogenic bone protection mediated by icariin and EXD (Figure 7.1). Icariin and EXD perhaps suppress the inhibitory effect of ER-α36 and GPER in bone formation by reducing their expressions, formation of signaling complexes on the cell membrane, and cellular translocation, thereby, promoting ER-α66-dependent rapid and genomic estrogen signaling in osteoblasts. In addition, the pOC-ERαKO mice model allowed us to understand the ER-α66-dependence of EXD and ER-α66-independence of icariin during bone formation (figure 7.2). It is also found that the ER-α66 expression and E_2 level might determine the action of ERs. ER-α66 perhaps worked dominantly in the normal physiological E_2 level in osteoblast while ER-

α36 is highly sensitive to E_2 and works dominantly in the postmenopausal E_2 level in osteoblasts expressing a low level of ER-α66.

Table 7.1 Summary of key findings of study about the roles of ER-α36 and GPER in rapid estrogenic effect of icariin and EXD in bone

	Tissue responsiveness			Bone protective effect			Signaling transduction	
	ER protein expression	ER protein expression	ER-α36 promoter	MTS assay	mRNA expressions	Rapid estrogen signaling	Signaling activation	Signaling complexes
Tissues and cells	Tibia head from rats(vs. OVX)	MC3T3-E1 and MG-63 cells	HEK293 cell	MC3T3-E1 and MG-63 cells	MC3T3-E1 and MG-63 cells	MC3T3-E1 and MG-63 cells	MC3T3-E1 cells and rBMSCs	rBMSCs
E_2 treatment	ER-α66: ↓ ER-α36: ↓ GPER : ↓	ER-α66: ↑ ER-α36: ↓ GPER : ↓	↓	G15: NC	G15: OPG ↑	G15: pAKT, pERK, pER (Ser167) ↑	ER-α36 and GPER : move to call edge ER-α66: move to nucleus	ER-α66/GPER: NC ER-α66/ER-α36: ↓ ER-α66/shc: ↑ ER-α66/c-src: NC
				GPER KD: NC	GPER KD: NC	GPER KD: pAKT ↑		
				ER-α36 OP: ↓	ER-α36 OP: OPG ↓, RANKL ↑	ER-α36 OP: NC		
Icariin treatment	ER-α66: ↓ ER-α36: ↓ GPER : ↓	ER-α66: ↑ ER-α36: ↓ GPER : ↓	↓	G15: ↑	G15: OCN, OPG↑	G15: p-ERK, pER (Ser167) ↑	ER-α36 and GPER : move to call edge ER-α66: move to nucleus	ER-α66/GPER: NC ER-α66/ER-α36: ↓ ER-α66/shc: ↑ ER-α66/c-src: ↓
				GPER KD: ↑	GPER KD: OPG ↑	GPER KD: pAKT, pER (Ser167) ↑		
				ER-α36 OP: ↓	ER-α36 OP: ALP, OCN ↓, RANKL ↑	ER-α36 OP: pAKT, pER (Ser167) ↓		
EXD treatment	ER-α66: ↓ ER-α36: ↓ GPER : ↓	ER-α66: ↑ ER-α36: ↓ GPER : ↓		G15: NC	G15: OCN, OPG↑	G15: pERK ↑		
				GPER KD: NC	GPER KD: NC	GPER KD: pAkt, pER (Ser167) ↑		
				ER-α36 OP: ↓	ER-α36 OP: OPG ↓	ER-α36 OP: pAkt, pER (Ser167), pER (Ser118) ↓		

GPER KD: GPER knockdown, ER-α36 OP: ER-α36 Overexpression, ↑: increase, ↓: decrease, NC: no significant change

Table 7.2 Summary of key findings of study about the ER-α66-dependency and involvement of ER-α3 and GPER in estrogenic effect of icariin and EXD in bone of pOC-ERαKO mice

		Long bone and spine			Tibia head		Primary osteoblast	
		Distal femur	Proximal tibia	Lumbar spine (L4)	ER protein expression	mRNA expression	MTS assay	Rapid estrogen signaling
pOC-ERαKO mice	Control	Femur length: ↓ Trabecular BMD: ↓ Cortical properties: ↓	Tibia length: NC Trabecular BMD: ↓ Cortical properties: ↓	Trabecular BMD: ↓ Cortical properties: ↓	ER-α66: ↓ ER-α36: ↑ GPER: ↑	OPG ↓	↓	pER (Ser118) ↓
	E_2						(10^{-8}M) ↓ (10^{-12}M, 10^{-10}M) NC	NC
	Icariin						NC	pERK↓
	EXD						NC	pERK↓

↑: increase, ↓: decrease, NC: no significant change

7.2 Limitation and future plans

The present study provided valuable scientific evidence explaining the mechanisms behind phytoestrogen and TCM in bone, however, the following limitations should be noted:

1. Transient transfection and siRNA were performed in *in vivo* study. The efficiency of ER-α36 overexpression and GPER knockdown might vary in each transfection. Also, the remaining GPER level was found in cell models as siRNA was transfected. Thus, the finding might not be accurate enough to fully reflect the roles of ER-α36 and GPER in bone formation.
2. ER-α-positive animal cell lines and pOC-ERαKO mice were utilised. Due to complicated crosstalk amongst ERs, it is hard to individually study the roles of each ERs in ER-α-positive cell lines, which originally express ER-α36, ER-α66, and GPER. Also, the knockout of ERα in pOC-ERαKO mice is limited to flanked exon 3. There might have other ERs domain remaining in the genome, which might interact with ERs upon treatment.
3. Although we showed that ER-α36 and GPER take part in rapid estrogen signaling mediated by icariin, the direct target of icariin is still unknown.

With the above limitation, there are few suggestions in future follow-up study.

1. Using Crispr-Cas9 system to perform complete knockout or stable overexpression in Cells. It could also establish a ER-α-negative cell model.
2. Generating a pOC-ERαKO mice by flanking different functional domain with loxP sites, or using conditional neo cassette, which allows longer cut sequence, to cut out ERα in mice.
3. Performing competitive binding assay to confirm if icariin binds to ER-α36 or GPER.

7.3 Conclusion

In conclusion, we demonstrated the unique mechanisms behind the bone protection of E_2, icariin, and EXD. Unlike physiological level E_2, icariin and EXD could bypass the classical estrogen receptor, ER-α66, to promote bone formation. Based on the present results, we suggested that two novel estrogen receptors, ER-α36 and GPER, might participate in bone protection by icariin and EXD. They seem to be negative regulators in bone formation and rapid estrogen signaling induced by icariin and EXD. Particularly, ER-α36 might crosstalk with GPER to inhibit the actions of ER-α66. Moreover, the functional role of ERs might depend on the estrogen level and the expression level of ER-α66. ER-α66 might work dominantly in osteoblasts at normal physiological E_2 level; while ER-α36 is highly sensitive to E_2 and works dominantly in osteoblasts at postmenopausal E_2 level.

This study elucidates the crosstalk between classical ER-α66 and novel ERs in bone formation and increases our understanding of the rapid estrogen signaling induced by icariin and EXD in which ER-α36 and GPER are potential therapeutic targets.

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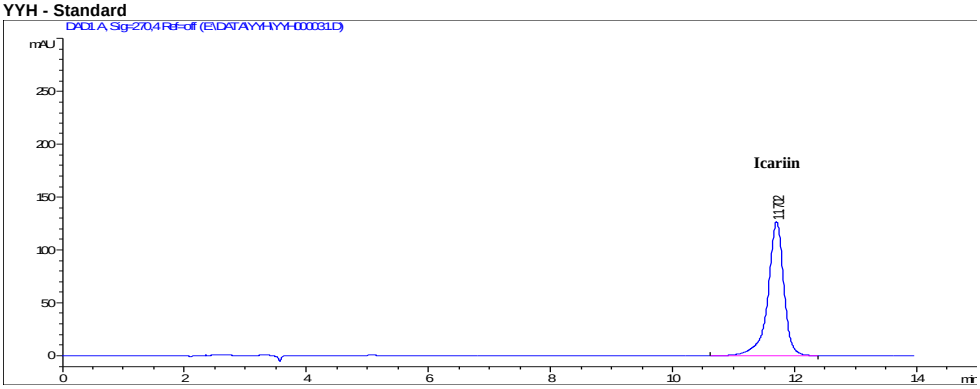
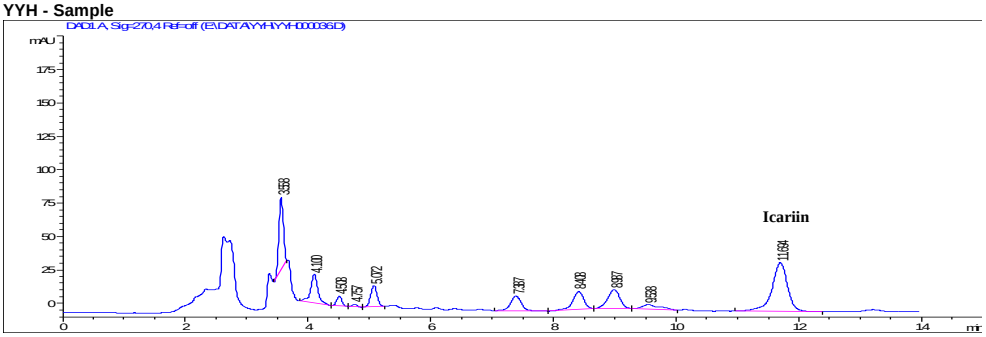
Appendices

A. Yinyanghuo (YYH): *Herba Epimedii*

YYH - Standard

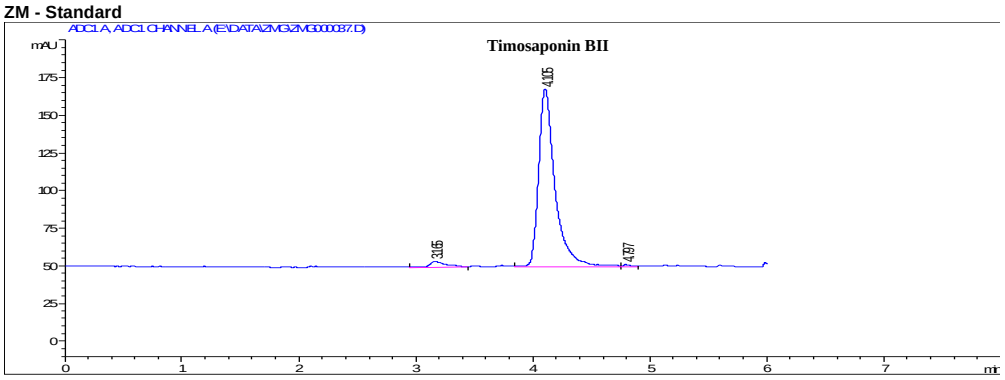


YYH - Sample



B. ZM (Zhimu): *Rhizoma Anemarrhenae Bunge*

ZM - Standard



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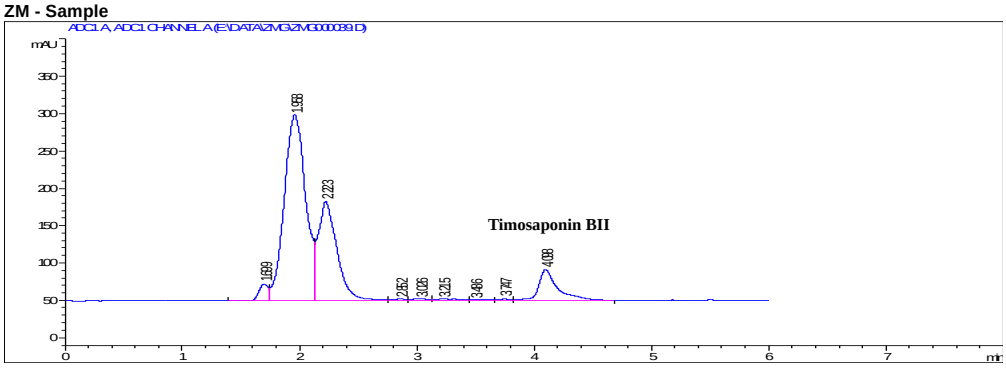
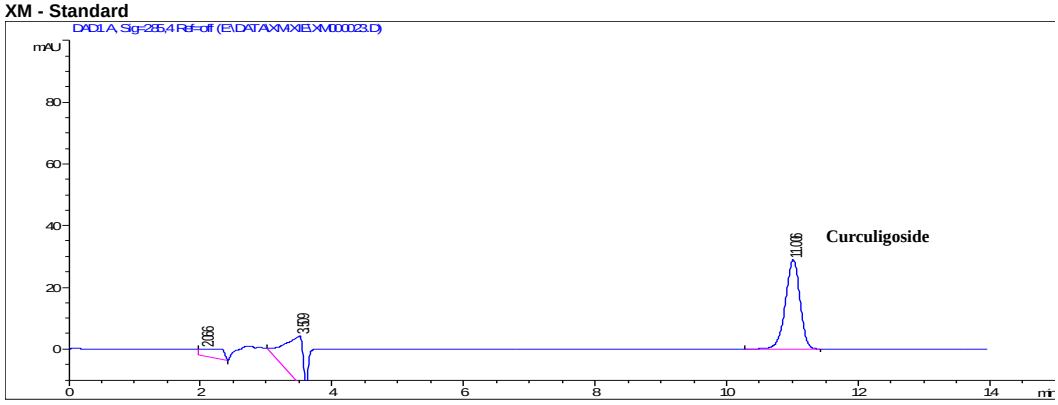


C. XM (Xianmao): *Rhizoma Curculiginis*

XM - Standard



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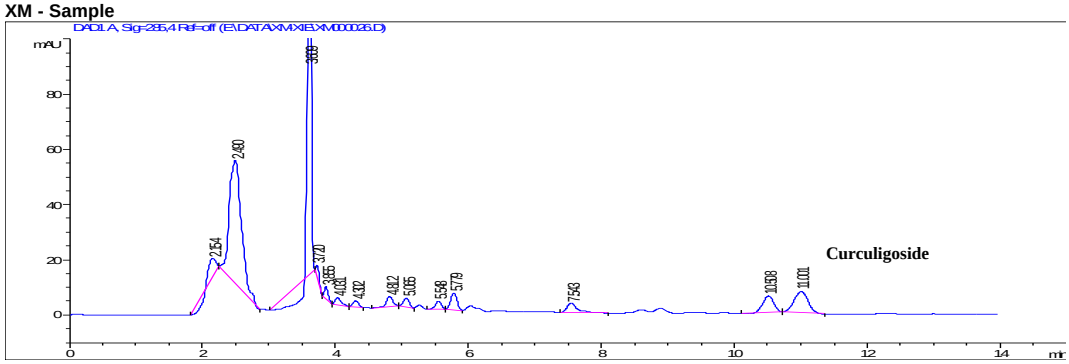
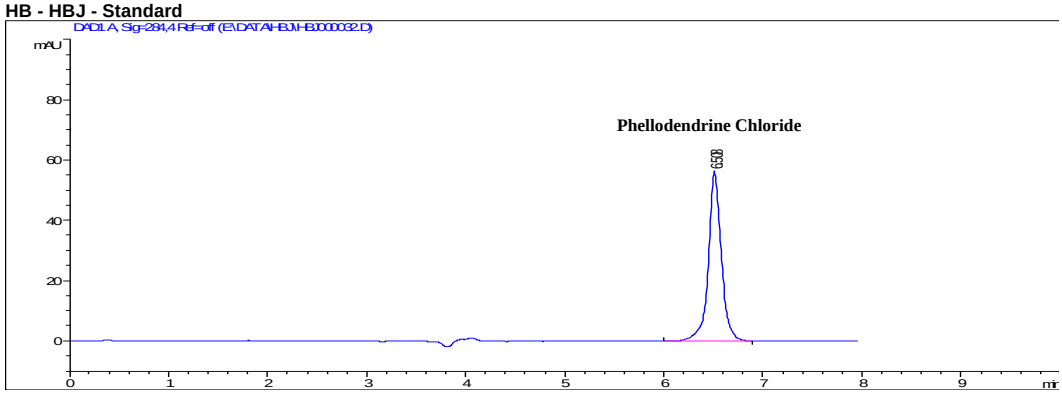


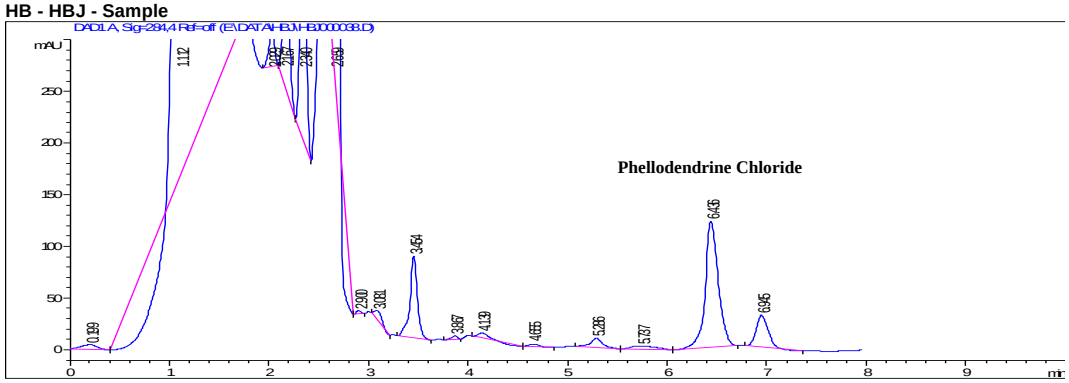
D. HB (Huangbo): *Phellodendron amurense Rupr*

HBJ (Huangbo Jian): Phellodendrine Chloride, CAS#: 6873-13-8

HB - HBJ - Standard



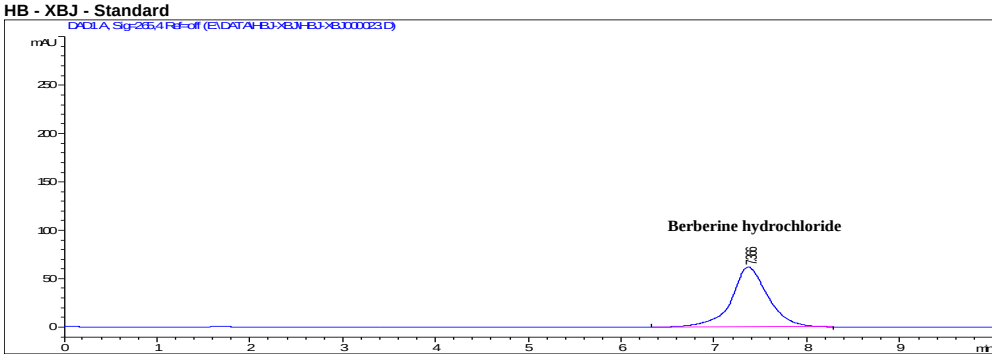
HB - HBJ - Sample



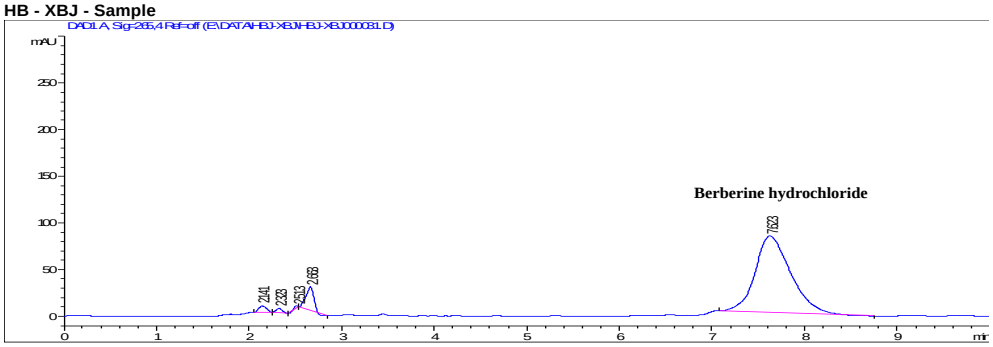
E. HB (Huangbo): *Phellodendron amurense Rupr*

XBJ (Xiaobo Jian): Berberine Chloride, CAS#: 2086-83-1

HB - XBJ - Standard

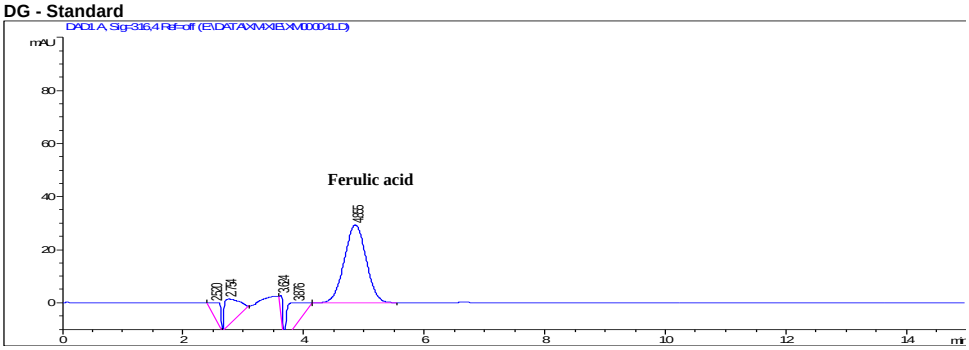


HB - XBJ - Sample

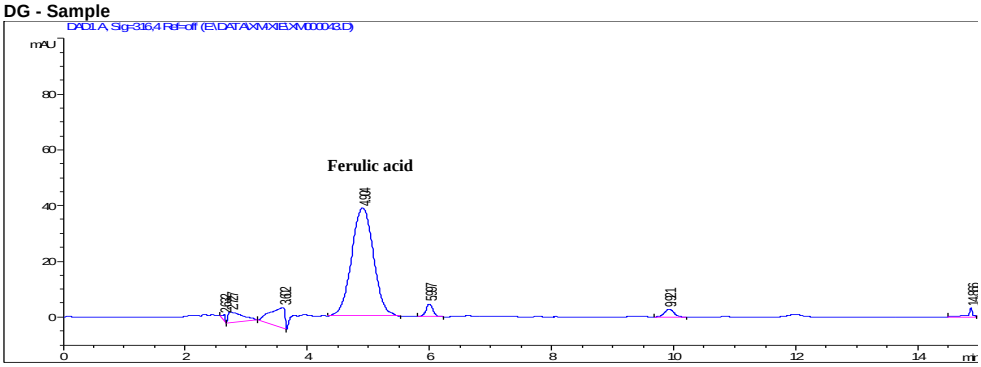


F. DG (Danggui): *Radix Angelicae Sinensis*

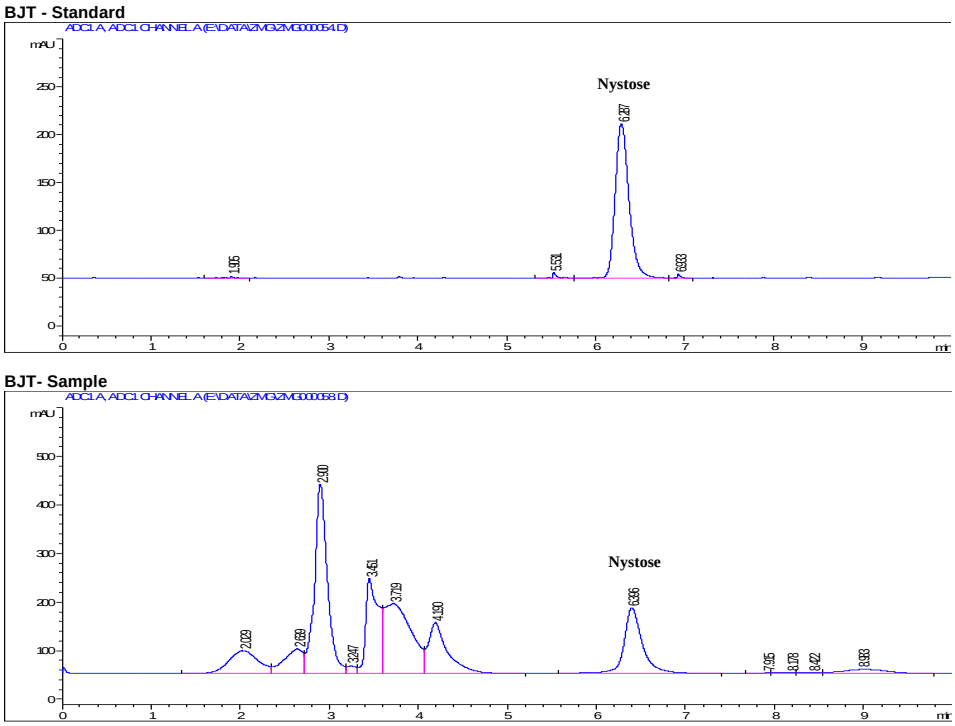
DG - Standard



DG - Sample



G. BJT (Bajitian): *Morindae Officinalis*



Appendix 1. Authentication of EXD raw herbs using HPLC. HPLC chromatograms of *Herba Epimedii* (A), *Rhizoma Anemarrhenae Bunge* (B) Rhizoma Curculiginis *(C)* Phellodendron amurense Rupr *(D, Radix Angelicae (E), Sinensis Morindae Officinalis.*

Appendix 2.

		HPLC results		Requirement (2017)
Herb	**Standard**	**ug/g**	**%**	**%**
Herba epimedii (HEP, Yin Yang Huo, 淫羊藿)	Icariin	5025.2822	0.5025	>0.5%
Curculigo Rhizoma (Xian Mao, XM, 仙茅)	Curculigoside	1436.0690	0.1436	>0.08%
Radix Morindae (Ba Ji Tian, BJT, 巴戟天)	Nystose	37801.9544	3.7160	>2.0%
Cortex Phellodendri (Huang Bo, HB, 黄柏)	Phellodendrine Chloride,	49578.2644	4.9578	>3%
	Berberine Chloride	18207.1940	1.8207	>0.34%
Angelicae Sinensis (Dang Gui, DG, 當歸)	Ferulic acid	1032.2760	0.1032	>0.05%
Rhizoma Anemarrhenae (Zhi Mu, ZM, 知母)	Timosaponin BII	34048.4045	3.4048	>3%

Appendix 3. Chemical constituents of EXD, HEP and RD extract by LC-MS

Extract	Herbs	Chemical marker	Contents (mg/g)
EXD	*Rhizoma Anemarrhenae Bunge* (Zhimu, ZM)	Timosaponin BII	-
	Rhizoma Curculiginis (Xianmao, XM)	Curculigoside	0.002
	Herba Epimedii (Yinyanghuo, YYH)	Icariin	1.605
	Phellodendron amurense Rupr (Huangbo, HB)	Berberine hydrochloride	1.814
		Phellodendrine Chloride	0.775
	Radix Angelicae Sinensis (Danggui, DG)	Ferulic acid	0.007
	Morindae Officinalis	Nystose	4.344

Note: "-" Trace amount