THE INVOLVEMENT OF ESTROGEN RECEPTOR (ER) AND G PROTEIN-COUPLED ESTROGEN RECEPTOR (GPR30) IN RAPID CELLULAR SIGNALING OF PHYTOESTROGENS IN OSTEOBLASTS

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The Involvement of Estrogen Receptor (ER) and G protein-coupled Estrogen Receptor (GPR30) in Rapid Cellular Signaling of Phytoestrogens in Osteoblasts

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A thesis submitted in partial fulfillment of the

requirements for the degree of Master of Philosophy

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Certificate of Originality

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(Name of student)

Abstract

Naringin, Icariin and Genistein are phytoestrogen found in citrus fruits, Herba Epimedii, and legumes, respectively. *In vivo* studies reported that these three phytoestrogens mimicked estrogen in exerting anabolic effects on bone, making them potential alternatives for the prevention and treatment of osteoporosis. Recently, estrogen was found to be not only acting on genomic signalling by classical nuclear estrogen receptors, ERα and ERβ, but also acting on rapid cellular signalling that involved both ERs and a novel membrane-bound estrogen receptor, GPR30. This study was designed to investigate the anabolic effects of each phytoestrogen on osteoblastic functions and the underlying signaling pathways by which they exert their effects.

Rat osteoblastic UMR106 cells were treated with naringin, icariin and genistein for 24hrs or 48 hrs to study their effects on cell proliferation (MTS assay), differentiation (ALP assay) and modulation of osteoclastogenesis (OPG/RANKL mRNA expression ratio). The results indicated that naringin and icariin significantly increased cell proliferation rate, stimulated alkaline phosphatase (ALP) activity, and up-regulated OPG/RANKL ratio in UMR106 cells. Genistein exerted stimulatory effect at a lower concentration (10^{-9}M to 10^{-12}M) and inhibitory effect at a higher concentration (10^{-5}M) on UMR106 cell proliferation and differentiation, but did not alter the OPG/RANKL ratio. The promoting effects of all three phytoestrogens on cell proliferation and differentiation could be abolished by co-treatment with ER antagonist ICI182780 as well as with GPR30 specific antagonist G15, indicated that both ER-mediated genomic pathway and GPR30-mediated cellular signaling might be involved in regulating the action of phytoestrogens

in osteoblasts. However, G15 did not affect the modulation of OPG/RANKL ratio by phytoestrogens, suggesting that GPR30 has limited role in modulation of osteoclastogenesis by osteoblasts.

As the effects of these compounds in UMR106 cells were similar to that of 17β-estradiol, the molecular actions of each compound via estrogenic signalling pathways in UMR106 cells were investigated. To explore the action of phytoestrogens via ER-mediated signalling pathway, the binding ability of each compound with classical ERs, their abilities to induce ERE-dependent transcription and ligand-independent phosphorylation of ERα at Ser118 in UMR106 cells were examined. Our results indicated that naringin and icariin showed no binding affinity to both ERα and ERβ, while genistein bound both ER isoforms but showed stronger affinity to ERβ. Genistein, but not naringin and icariin, was able to induce ERE-luciferase activity via both ERα and ERβ in UMR106 cells, with stronger induction by ERβ. However, all three compounds were able to significantly induce ERα phosphorylation at Ser118, suggesting that their actions might involve ligand-independent activation of ER in UMR106 cells instead of direct binding with ER to induce ERE transcription.

Since the ligand-independent activation of ER is mediated by rapid estrogenic signalling cascades, the molecular actions of phytoestrogens in UMR106 cells via MAPK signalling pathway, PI3K/Akt pathway and intracellular cAMP-dependent signaling were examined. Our results demonstrated that all three compounds were able to activate MAPK signalling through stimulating phosphorylation of ERK-1/2 as rapid as 5 minutes

without altering MEK phosphorylation. Whereas 17β-estradiol and genistein inhibited the basal phosphorylation of Akt and downregulated protein expression of Akt and PI3K, naringin and icariin were able to activate Akt in the PI3K/Akt signaling. On the other hand, instead of stimulating cAMP production, treatment with 17β-estradiol and all compounds for 10 minutes demonstrated no effect or even weakly inhibit the intracellular cAMP content and unable to induce CRE-dependent gene transcription in UMR106 cells.

To investigate the role of GPR30 as an estrogen receptor in the actions of phytoestrogens in UMR106 cells, G15 were pre-incubated for 20 minutes before treatment with the compounds. While pretreatment with ICI182780 completely abolish the stimulation of ERE-dependent transcription by genistein via both ERα and ERβ, pretreatment with G15 only partially inhibited ERE-dependent transcription via ERα but not ERβ, indicating the presence of interrelationship between ERα and GPR30. Surprisingly, pretreatment with G15 not only failed to abolish the stimulating effects of phytoestrogens on ERK-1/2 phosphorylation, ERα phosphorylation at Ser118, and Akt phosphorylation, but elevated their basal phosphorylation as well as promoted the effects of phytoestrogens treatment. The results suggest that the estrogenic signaling of phytoestrogens in UMR106 cells does not require GPR30 activation and that GPR30 might play a different role in regulating the molecular actions of estrogenic compounds in osteoblasts.

In conclusion, naringin, icariin and genistein demonstrated anabolic effects on osteoblast functions, and their estrogenic actions are ER-dependent and regulated by rapid cellular signalling via MEK/ERK and PI3K/Akt activation. GPR30 is involved in the effects of phytoestrogens but has a distinct role in the estrogenic signalling mechanism in osteoblast.

List of Publications

1. H.H. Xiao, C.Y. Fung, S.K. Mok, K.C. Wong, M.X. Ho, X.L. Wang, X.S. Yao, M.S. Wong (2013) "Flavonoids from *Herba epimedii* selectively activate estrogen receptor alpha (ERα) and stimulate ER-dependent osteoblastic functions in UMR106 cells." *Journal of steroid biochemistry and molecular biology.* Submitted.
2. M.X. Ho and M.S. Wong (2013) "Estrogenic signalling pathways of naringin and genistein in rat osteoblastic UMR106 cells". *The IOF Regionals – 4th Asia-Pacific Osteoporosis Meeting*, Hong Kong. Submitted.
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4. M.X. Ho, X.L. Dong, K.C. Wong and M.S. Wong (2012) "Involvement of GPR30-mediated signalling in the estrogenic effects of flavonoids on rat osteoblastic UMR106 cells." *2012 Annual Meeting of the American Society for Bone and Mineral Research*, Minneapolis, USA. Abstract MO0217.
5. X.L. Dong, S.S. Gu, Q.G. Gao, M.X. Ho, H.T. Feng, M.S. Wong and L.Y. Yan (2012) "Effects of ethanol and water extracts of *Fructus ligustri Lucidi* on vitamin D metabolism and intestinal calcium absorption in mature ovariectomized (OVX) rats." *2012 Annual Meeting of the American Society for Bone and Mineral Research*, Minneapolis, USA. Abstract SU0415.

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List of Abbreviations

AF-1/AF-2	Active function-1/2
ALP	Alkaline Phosphatase
BMD	Bone mineral density
BMP	Bone morphogenic protein
BMSC	Bone marrow stromal cells
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophosphate
cDNA	complementary DNA
CRE	cyclic AMP response element
DBD	DNA binding domain
DEXA	Dual energy X-ray Absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
E2	17β-estradiol
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptors
EIA	Enzyme immunoassay
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FPPS	Farnesyl pyrophosphate synthase
GPCR	G-Protein coupled receptor
GPR30	G-Protein Receptor 30
GPER1	G protein-coupled estrogen receptor 1
HB-EGF	Heparin-binding EGF-like growth factor
HR	Hazard ratio
HRT	Hormone Replacement Therapy
ICI	ICI182780,7a-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] -estra-1,3,5(10)-triene-3,17b-diol
IGF-1	Insulin-like growth factor-1
IL	Interleukin
JNK	Jun N-terminal kinase

LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulphophenyl)-2H-tetrazolium
mRNA	messenger RNA
NF-kB	Nuclear factor kappa B
OPG	Osteoprotegrin
OVX	Ovariectomized
PBS	Phosphate buffered saline
PLB	Passive lysis buffer
PI3K	Phosphoinositide 3-kinase
PKA/PKC	Protein kinase A/ Protein kinase C
PMS	Phenazine methosulfate
pNPP	p-nitrophenylphosphate
PTH	Parathyroid hormone
qPCR	quatitative polymerase chain reaction
RANKL	Receptor activator of NF-κB ligand
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrlamide gel electrophoresis
SEM	Standard error of mean
SERM	Selective estrogen receptor modulator
SRE	Serum response element
sFBS	stripped- Fetal Bovine Serum
TTBS	Tris-Buffered Saline with Tween 20
WHO	World Health Organization
WHI	Woman's Health Initiative

Chapter 1

Background and Introduction

1.1 Osteoporosis

Osteoporosis is a prevailing skeletal disorder which affects a vast population around the world. A global research by Oden et al collected data from 58 countries and estimated that in year 2010 alone, around 2.7 million new cases of osteoporosis-associated hip fractures incidence occurred worldwide (Odén et al. 2013). It is regarded as one of the most common causes of disability and billions of dollars were spent every year for the treatment as well as nursing-home care of osteoporotic fractures (Genazzani 2006);(Burge et al. 2007). It is widely speculated that with the increase in life expectancy and the fast growing population, a larger socioeconomic burden is inevitable for the treatment of osteoporosis. In fact, it is expected that following the increase of individual aged 65 years and more from 323 million to 1555 million by 2050, the worldwide annual costs for treatment of hip fractures will rise to approximately US$131.5 billion (Harvey et al. 2010).

Osteoporosis is a disease characterized by decreased bone quality and micro-architecture damage of bone tissue, leading to an increased risk of bone fracture especially in the hip, spine and wrist (Cooper et al. 2005). Bone strength was determined by both bone quality and bone mineral density (BMD). By exploiting various bone densitometric techniques, particularly dual energy X-ray absorptiometry (DEXA), BMD (grams of mineral / area or volume of bone) is used as a golden standard for the prediction of the risk of osteoporosis and hip fracture. A value of BMD larger than 2.5 S.D. (T-score) below mean of a young adult is diagnosed as osteoporosis as defined by World Health Organization (WHO) (Cooper et al. 2005; Genazzani 2006).

Bone is a metabolically active tissue that is constantly formed, removed and

replaced throughout one's lifespan by undergoing the bone remodeling cycle **(Figure 1.1)**. Basically, bone cells consist of 3 major cell types, namely osteoblasts, osteoclasts and osteocytes (Cooper et al. 2005). The former 2 cell types operate simultaneously and interact with each other, forming a basic multi-cellular unit (BMU) to regulate bone density by maintaining the dynamic homeostasis between bone formation (osteoblasts) and resorption (osteoclasts) (Turner et al. 2001; Harada et al. 2003; Krishnan et al. 2006).

Osteoblasts are bone-forming cells derived from mesenchymal progenitors. They are responsible for synthesis of bone collagen, production of bone-related proteins such as alkaline phosphatase (ALP), bone matrix mineralization, modulation of bone resorption activity and regulation of many other bone functions (Harada et al. 2003). In contrast, hematopoietic lineage-derived osteoclast cells are responsible for bone resorption by producing lysosomal enzymes to seal off and resorb the bone matrix under acidic environment on bone surface (Manolagas et al. 1995). For maintaining the bone homeostasis and a stable BMD in an individual, the interaction and balance of activities between osteoblasts and osteoclasts are essential. The disruption of this bone homeostasis, either the reduce of osteoblastic function or the excess osteoclasts activity will affect the BMD, leading to the development of osteoporotic-related diseases (Genazzani 2006).

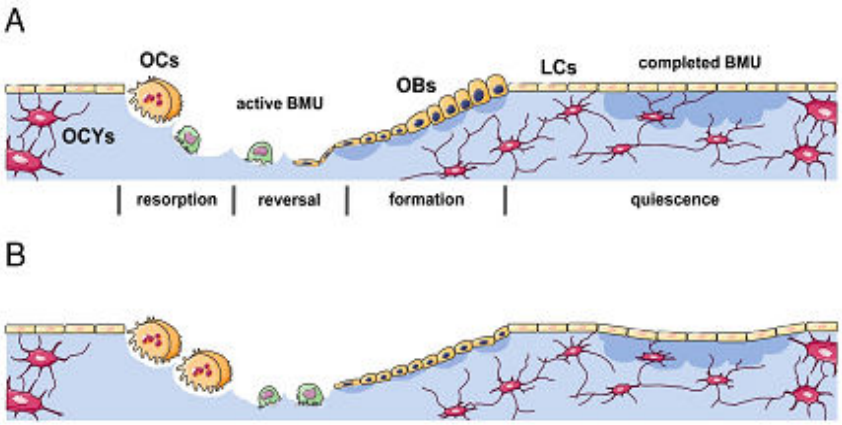


Figure 1.1: Bone remodeling cycle under normal conditions and in osteoporosis. In the bone remodeling cycle, (A) Bone resorption is controlled by osteoclasts (OCs) followed by formation of new bone matrix by osteoblasts (OBs). The interactions between the remodeling functions of these two cell types form an active basic multi-cellular unit (BMU) which is essential in maintaining bone homeostasis.
(B) In osteoporosis, the increase in bone resorption rate and the decrease in bone formation lead to an imbalanced bone remodeling activity and subsequent bone loss. (Baron et al. 2012)

1.2 Estrogen Deficiency and Postmenopausal Osteoporosis

Among all the risk factors associated with this health threat, the population of post-menopausal women is found to have a much higher prevalence of osteoporosis. Study found that in population aged over 50, almost 40% of women would be at risk of suffering osteoporosis-associated fractures in their lifetime compared to 13% in men (Melton et al. 2005). The development of involutional osteoporosis in women at post-menopausal stage is highly associated with estrogen deficiency (Type I osteoporosis) and age-related bone loss(Type II osteoporosis) (Riggs et al. 1998).

Figure 1.2 demonstrates the trends of bone mass in association with aging

particularly in women. The continuous, steady bone growth of a woman reaches its peak after the adolescent phase, follows by a transient stability period in BMD spanning from 30 to 40 years old. The rate of bone resorption surpasses bone formation following the increase in age after 40, leading to loss in the bone mass. The decrease in BMD is greatly accelerated at the start of postmenopausal period, leading to a high risk in development of osteoporosis and increasing the risk of bone fracture (Riggs et al. 1998; Kubota et al. 2009). **(Figure 1.2)**

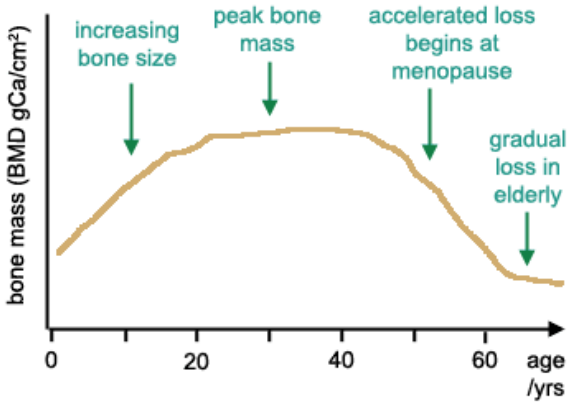


Figure 1.2: Changes in bone mass over the lifetime of women(Harada et al. 2003)

In contrast with men, bone loss in post-menopausal women involves two main phases: the acceleration phase and the late phase. It was reported that over the first decade following menopause, woman can experience up to 20%-30% bone density loss in cancellous bone, follow by a continuous bone loss at the late slow phase for the subsequent lifetime until death (Riggs et al. 1998). The steep decrease in bone density in women after menopause results from the loss of ovarian function which leads to impaired production and deficiency of the sex steroid, estrogen. 17β-estradiol (E2), the endogeneous form of estrogen, is pivotal in modulating bone homeostasis and regulating

the bone turnover. E2 suppresses bone resorption via the direct inhibition of osteoclast functions and their differentiation ability (Oursler et al. 1991; Riggs et al. 1998) , while promotes bone formation via stimulation of osteoblastogenesis and osteoblastic cells differentiation (Genazzani 2006).

Hence, the postmenopausal state in women creates an estrogen deficiency condition in which the ability of estrogen in regulating the bone homeostasis is no longer effective. The deficiency of E2 causes concurrent occurence of increased bone resorption and decreased bone formation, leading to the imbalance in bone remodeling and continuous decrease of bone density, which eventually leads to the development of involutional osteoporosis. **(Figure 1.3)**

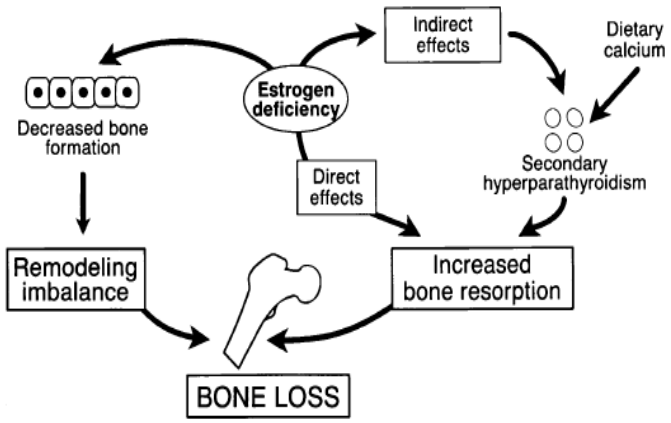


Figure 1.3: Model for relationship between estrogen deficiency and bone loss in postmenopausal woman(Riggs et al. 1998).

1.3Current Therapies for Osteoporosis

1.3.1 Hormone Replacement Therapy

The most direct method to counteract the effects of estrogen deficiency in postmenopausal osteoporosis is the use of Hormone or Estrogen Replacement Therapy (HRT). HRT has been used widely as a promising prescription for the prevention and treatment of postmenopausal osteoporosis for more than 25 years (Glazier et al. 2001; Huot et al. 2008). Clinical studies demonstrated that the therapy led to an overall increase in BMD by 5-10% among postmenopausal subjects (Lindsay et al. 1980; Bush et al. 1996; Cooper et al. 2005), reduced risk of osteoporosis by 50% and relieved other menopause-related symptoms, suggesting that HRT is an effective approach against osteoporosis (Grady et al. 1992).

Nonetheless, the major drawback for HRT is that it causes several detrimental side effects that might pose potential health risk to the patients. Women's Health Initiative (WHI) provided strong evidence in 2002 that HRT is associated with higher risks in developing breast cancer (estimated hazard ratios, HR=1.26 after 5-10 years of use), stroke (HR=1.41), coronary heart disease (HR=1.29) and other diseases (Glazier et al. 2001; Rossouw et al. 2002) **(Figure 1.4)**. Based on the clinical results, WHI strongly discourages the use of HRT as a long term first line therapy in the prevention and treatment of osteoporosis as the overall health risks of HRT outweighed its benefits.

Since then, there was a significant decline in the prescription of HRT following the change in clinical guidelines and population preference. For instance, a 39% decrease in French women population who took up the HRT prescription was observed between 2004 to 2006 (Huot et al. 2008). Currently, the effort of physicians in treatment of

osteoporosis has been mainly focused on searching alternative medications that have better safety profiles and yet still demonstrate considerable potency in preventing bone loss or promoting bone formation.

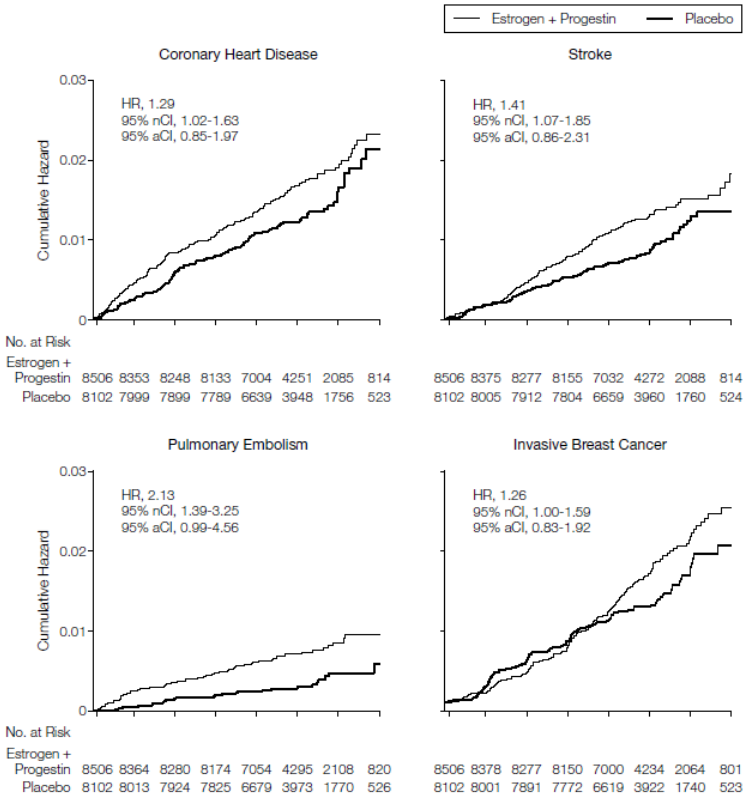


Figure 1.4: WHI estimation of cumulative hazards for several clinical outcomes (Rossouw et al. 2002).

1.3.2 Alternative therapeutic approaches for osteoporosis

Currently, the approved therapeutic treatments and treatments under development for osteoporosis focus on two main categories: Antiresorptive therapies and bone-anabolic drugs. The strategy of antiresorptive drugs lies on the restoration of bone mineral density by reducing the bone resorption rate and slowing down the whole bone turnover. Hence, the treatment target for these antiresorptive drugs is mainly on osteoclasts. For instance, bisphosphonates, the most commonly used antiresorptive

treatments for postmenopausal osteoporosis, reduce osteoclastic activity and survival by the inhibition of the enzyme farnesyl pyrophosphate synthase (FPPS) which is essential for osteoclast functions (Russell et al. 2008). However, a major drawback of these antiresorptive drugs is that they might lead to a lower bone turnover state in which the rate of bone formation decreases following the reduction of bone remodeling activity (Baron et al. 2012).

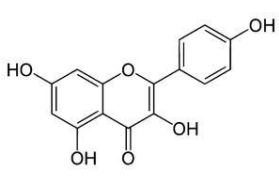
Instead of preventing bone loss, bone-anabolic agents aim at building up bone mass and improving the bone micro-architecture by preferential stimulation of osteoblasts activity over osteoclasts. Currently, the only approved bone-anabolic drugs on the market against osteoporosis are the injectable forms of recombinant human parathyroid hormone (rhPTH1-34, Teriparatide).While continuous exposure to PTH lead to catabolic effects on bone, studies found that intermittent administration of PTH improved bone properties, increased bone turnover markers and reduced incidence of fractures (Neer et al. 2001; Greenspan et al. 2007) The effects were attributed to the ability of this hormone to increase the number and activity of osteoblasts and delay the osteoblastic apoptosis (Jilka 2007; Kanis et al. 2013). On the other hand, recent understanding of the role of Wnt-dependent signaling pathway as a major regulating factor in osteoblastic differentiation and bone formation lead to the development of a range of antibodies against the endogenous inhibitors of Wnt signaling, sclerostin and Dkk-1(Rachner et al. 2011). While the use of these anabolic drugs present as potential therapeutic targets for treatment of osteoporosis, the relatively high cost of production, inconvenient route of administration, the safety concerns in particular the association of Wnt signaling to tumors development will require further clinical investigation (Baron et al. 2012).

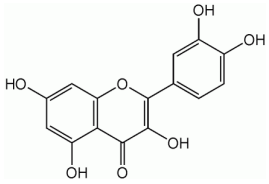
Table 1.1: Development of alternative approaches for osteoporosis treatment and their drawbacks

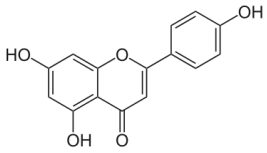
Category	Drug Class	Mode of action	Adverse effects/ Drawbacks	References
Anti-resorptive Drugs	**Bisphosphonates**	Strong affinity to hydroxyapatite in bone, reduce osteoclast activity and recruitment, induce osteoclast apoptosis	Atypical subtrochanteric fractures, osteonecrosis of the jaw	**(Black et al. 1996) (Silverman et al. 2007) (Khosla et al. 2007) (Watts et al. 2010)**
	Denosumab	human monoclonal antibody against RANKL, inhibits osteoclastogenesis and osteoclast differentiation.	Discontinuation lead to rapid increase of bone-turnover markers	**(Lewiecki et al. 2007) (Miller et al. 2008) (Cummings et al. 2009)**
	Calcitonin	endogenous polypeptide hormone, depress osteoclastic bone resorption	Efficacy remains questionable, relatively high cost	**(Civitelli et al. 1988) (Cranney et al. 2002)**
	Odanacatib	Selective cathepsin K inhibitor，prevent collagen degradation and bone breakdown.	No observable side-effects reported yet	**(Eisman et al. 2011) (Bone et al. 2010)**
Bone-anabolic drugs	**rhPTH (1-84 PTH) Teriparatide (1-34 N terminal fragments)**	Intermittent administration increases proliferation and activity of osteoblasts, improves osteoblastic survival	Hypercalcaemia, nausea, headache, dizziness, relatively high cost	**(Neer et al. 2001) (Jilka 2007) (Greenspan et al. 2007)**
	Inhibitors of Wnt antagonists	Antibodies against sclerostin and dickpoff1, activation of canonical Wnt signaling pathway	Oncogenic concerns, unclear safety profile	**(Heiland et al. 2010) (Rachner et al. 2011)**

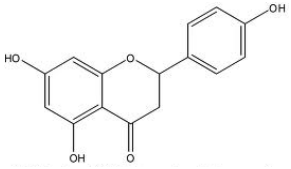
1.4Phytoestrogens

Flavonoids

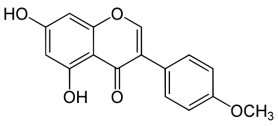

Kaempferol

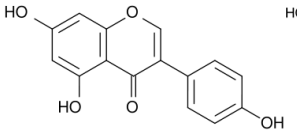

Quercetin

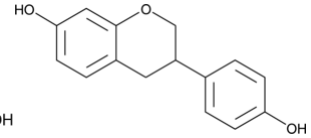

Apigenin

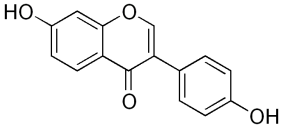

Naringenin

Isoflavonoids

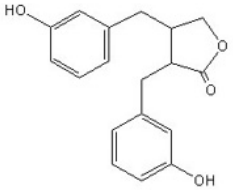

Biochanin A

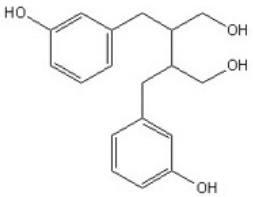

Genistein


Equol

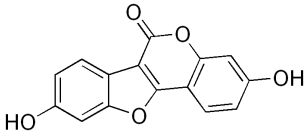

Daidzein

Lignans


Enterolactone


Enterodiol

Coumestanes


Coumestrol

Stilbenes

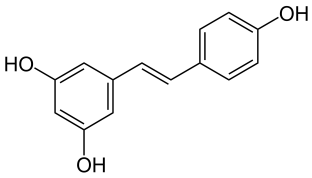

Resveratrol

Figure 1.5: Classification of phytoestrogens (adapted and modified from (Moutsatsou 2007))

1.4.1 Structural classification of phytoestrogens

There is a growing interest in studying phytoestrogens on these years as a potential replacement of the traditional HRT. Phytoestrogen is a diverse group of naturally existing phenolic compounds isolated from plants, fruits and vegetables which structurally or functionally mimic the effects of estrogen. Their structural similarity with the endogenous form of estrogen, 17β-estradiol (E2) confers them a certain degree of estrogen-like properties. Their 2-phenylnaphtalene-like structures enable them to either

bind estrogen receptors, despite the fact that their affinities are generally weaker in compare to the endogeneous E2, or functionally mimic E2 in the intracellular signaling cascades (Glazier et al. 2001). The effects of phytoestrogens can be estrogenic and also anti-estrogenic. Their behaviors seem to depend on the concentrations, their interaction with different estrogen receptors and the responses are tissue-specific (Martinez-Campos et al. 1986). Collectively, phytoestrogens were found to possess some beneficial estrogenic effects on the treatment against cardiovascular diseases, high cholesterol level, cancers development, osteoporosis and various menopausal symptoms (Tham et al. 1998; Ososki et al. 2003). Based on their structures, phytoestrogen are categorized into several main classes: the flavonoids, lignans, coumestans and stilbenes **(Figure 1.5).**

Flavonoids, which are the major class of phytoestrogens containing some 5000 compounds, could be further divided into several different subgroups, such as flavones, flavonols, flavanones, flavanols (cathechins), anthrocyanidins, isoflavones etc. They have a common structure of flavone backbone which is believed to be responsible for their estrogenic properties **(Figure 1.6)**. In the plant sources, they exist mainly in glycoside form (with the exception of cathechins), i.e. conjugation with sugars, and occasionally in aglycone form (Moutsatsou 2007). It was previously believed that only aglycones were able to be absorbed in human intestines as the hydrophilicity of the glycosides does not favor the absorption. However, a series of studies done by Hollman and colleagues on quercetin glucosides demonstrated not only that the glycoside forms showed high absorption in small intestines, but also that the sugar moiety attached in the flavonoids even improved the absorption (Hollman et al. 1995; Hollman et al. 1997; Hollman et al. 1999).

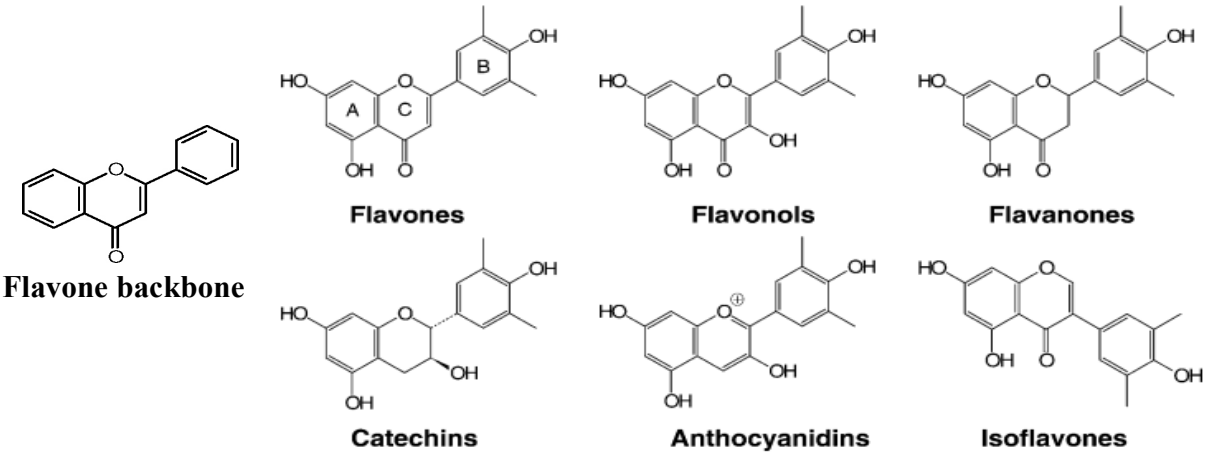


Figure 1.6: Molecular structure of flavone backbone and different subclasses of flavonoids. (Hollman 2004)

Among the different subgroups of flavonoids, isoflavones are the most frequently investigated compounds due to their well-known potency in mimicking E2 (Prossnitz et al. 2008). Unlike most flavonoids that are ubiquitously found in various plant sources, isoflavones exist predominantly in legumes, particularly in soy. Analysis of different soy products demonstrated high total isoflavones content in these foods, ranging from 0.1 to 3.0 mg/g (Setchell et al. 1999). Starting from the past 20 years, the soy isoflavones receives a great deal of interest as they are often associated with beneficial effects on cardiovascular diseases, osteoporosis and other hormone-related diseases including certain cancers (Messina et al. 1991; Anderson et al. 1999)

There are more than 1000 types of isoflavones, each of them possessing distinct estrogenic activity. Nonetheless, they share a common diphenolic structure which resembles the structures of synthetic estrogens such as diethylstilbesterol (Tham et al.

1998). The hydroxyl group in each of their phenolic ring and the similar distance between two hydroxyl groups enables them to bind directly to the estrogen receptors ERα and ERβ with different affinities, though preference to ERβ is observed. Among them, genistein and daidzein were the most abundant isoflavones in soy and soy products.

Lignans are another major class of phytoestrogens which are most abundant in flaxseed. When ingested, the plant lignans serve as precursors and then metabolized by the gut microflora in colon into mammalian lignans such as enterolactone and enterodiol. Studies found that lignans possess antioxidant activity and are associated with reduced risk of breast cancer (Chen et al. 2003) and myocardial infarction (Vanharanta et al. 1999). However, they only show very weak binding to estrogen receptors and transcriptional responses via ERs, suggesting that their effects involve nongenomic mechanisms (Mueller et al. 2004). Coumestans and stilbenes are isolated from clover and skin of grapes respectively. Both classes have been reported to bind ERs and possess estrogenic activities (Whitten et al. 1992; Gehm et al. 1997). Coumestrol, in particular, were reported to increase the mineralization of bone and inhibit bone resorption (Tsutsumi 1995).

In this study, we have investigated three plant-derived flavonoids, namely **Naringin, Icariin and Genistein**. Besides their potency in the prevention of osteoporosis which was suggested by previous studies (Suthar et al. 2001; Lu et al. 2006; Mok et al. 2010), these 3 phytoestrogens were originated from three distinct natural food sources: Naringin from citrus fruits, Icariin from herbs, and Genistein from legumes.

1.4.2 Naringin

Naringin (柚皮苷) is one of the flavonone glycosides found predominantly in citrus fruits. Various studies on potential health benefits of naringin suggested a positive role of this phytocompound on anti-ulcer effects, cardioprotection and protection against environmental toxins (Lu et al. 2006). Studies also associate naringin with anti-cancer effects such as inhibition of breast cancer cell proliferation (So et al. 1996). *In vivo* and *in vitro* studies on bone reveal that this flavonoid serves as a potent osteoprotective agent. Studies on the effect of naringin on retinoic acid-induced osteoporotic rats model showed that naringin treatment increased BMD in femur and higher calcium and phosphorus levels compared to the untreated group, suggesting that naringin has the potential to promote bone formation (Wei et al. 2007). Studies by our group as well as the others using ovariectomized (OVX) rat model also showed that naringin administration effectively reverse OVX-induced bone deterioration via increasing BMD, trabecular thickness and biomechanical strength of tibia (Pang et al. 2010; Li et al. 2013). In vitro studies also showed that the treatment with naringin significantly increased proliferation, osteogenic differentiation and bone formation markers in human bone mesenchymal stem cells (Dai et al. 2009), induced bone morphogenetic protein-2 expression and stimulate alkaline phosphatase (ALP) activities in murine preosteoblasts MC3T3 cells (Wu et al. 2008) and rat osteoblastic UMR106 cells (Pang et al. 2010). However, the mechanism of action in which naringin exerts its osteoprotective functions are still largely unknown and worth to be investigated.

1.4.3 Icariin

Icariin(淫羊藿苷) is a C-8 prenylated flavonol glycoside, a major flavonoid compound in Herba Epimedii which is commonly used as a Traditional Chinese Medicine for the treatment of cardiovascular disease and osteoporosis (Zheng et al. 1998; Gao et al. 1999). In the theory of Traditional Chinese Medicine, the bone quality is regulated by kidney and osteoporosis occurs when there is insufficiency in *kidney yang*. Thus, as a "kidney-tonifying" agent, the effect of Herba Epimedii could be closely associated with the overall bone health (Ma et al. 2011). Recent study by Meng at al isolated the active constituents of Herba Epimedii and investigated their bone anabolic functions using osteoblast-like UMR106 cells. The study found that icariin, among all other flavonoid compounds extracted, showed the highest potency in promoting the cell proliferation of UMR106 cells, indicating icariin played the leading role as the active ingredient in Herba Epimedii in treatment of osteoporosis (Meng et al. 2005). This finding was further supported by the studies of other groups that demonstrated the abilities of icariin to prevent bone loss in ovariectomized (OVX) mice models, stimulate proliferation and differentiation of primary osteoblast cells, and inhibit osteoclastogenesis and bone resorption activity(Huang et al. 2007; Nian et al. 2009; Mok et al. 2010). Studies also found that icariin effectively promotes osteogenic differentiation by up-regulating anabolic markers such as Runx2, BMP-2, SMAD4 and OPG in preosteoblastic MC3T3-E1 cells and primary osteoblasts (Zhao et al. 2008; Hsieh et al. 2010).These results indicated that icariin administration could be a potential approach in the prevention and treatment of postmenopausal osteoporosis.

1.4.4 Genistein

Abundant in legumes such as soy and beans, **genistein (染料木黃酮)** is perhaps one of the isoflavones which attain most attention of the investigators (Barnes et al. 1996). It was found that a lower indicence of osteoporosis in Eastern countries could be associated with a relatively rich soybean-based diet consumed in these population (Ishimi et al. 1999; Kurzer 2002), and it was demonstrated that genistein possesses the highest potency among soy isoflavones (Tissier et al. 2007). It was claimed that genistein has various pharmacological benefits including cardioprotection, antioxidant, cancer prevention and anti-cholesterol activity (Barnes et al. 1996; Anderson et al. 1998; Suthar et al. 2001). In vitro study demonstrated that treatment with genistein effectively prevent bone calcium loss in the OVX rats, suggesting that this isoflavone could be an bone-protective agent for the treatment of osteoporosis (Wang et al. 2006). The findings are further supported by in vitro studies which revealed that genistein could stimulate the osteoblastic differentiation via increasing ALP activity of human osteoblastic cells in dose-dependent manner (Chen et al. 2006). However, other studies suggested a potential catabolic effect of genistein on bone, showing that dietary genistein was able to inhibit the growth of prostate cancer bone tumor and suggesting that genistein may exert an inhibitory effect on bone metastasis (Li et al. 2004). It was proposed that genistein has biphasic effects on bone metabolism such that it stimulates osteogenesis and inhibits adipogenesis in mesenchymal stem cells at a lower concentration and the effects are opposite when it is administered at a higher dose (Dang et al. 2005; Reinwald et al. 2006);.

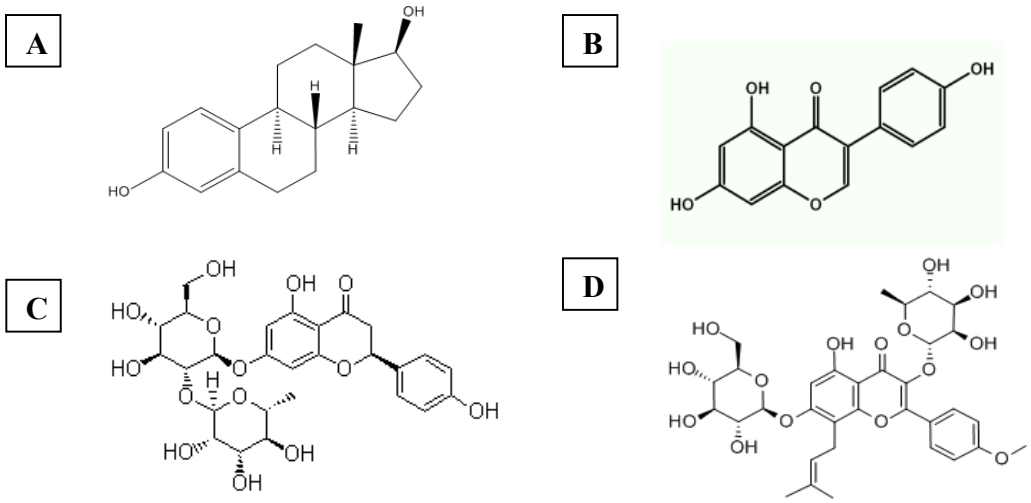


Figure 1.7: Structures of 17β-estradiol and the flavonoids.

(A)17β-estradiol (B) Genistein, an isoflavone (C) Naringin, a flavonone glycoside (D) Icariin, a prenylated flavonol glycoside

1.5 Pathways of Estrogen Signaling

1.5.1Classical Estrogen Receptors

The classical estrogen receptors exist in two subtypes, namely ERα and ERβ. In 1973, the first receptor for estrogen (ER, later named ERα) was discovered and characterized in breast cancer cells MCF7. Using competitive binding assays with [^{3}H]-17β-estradiol (E2), Brooke and colleagues demonstrated that this protein shown specific binding with E2 and was responsible to transport E2 from cytoplasm into the nucleus (Brooks et al. 1973). The subsequent identification of a second ER, ERβ in 1996 (Kuiper et al. 1996) leads to a more complicated picture of the actions of estrogen via these estrogen receptors in the cellular level, in which the two receptors might play different or synergistic roles in regulation of estrogen responsive genes. In the body, ERα is expressed predominantly in the reproductive organs (ovary, testis, uterus, mammary gland), while relatively higher expression of ERβ is found in brain, lung, prostate, bladder,

bone and thymus (Setchell et al. 1999). It was suggested that the difference between these two isoforms in terms of their tissue distribution and relative ligand binding affinity might help to explain the tissue-selectivity shown by actions of the selective estrogen receptor modulators (SERMs) and some ER agonists/antagonists (Kuiper et al. 1997).

ERα and ERβ are the members of the nuclear receptor superfamily, a large family of more than 150 ligand-regulated transcription factors which share a common structural architecture. The structures of both estrogen receptors contain six conserved functional domains labeled A to F according to their functional properties. Starting from the N terminal, the A/B domain is responsible for transcriptional regulation and contains an active function 1 (AF-1). C domain is known as the DNA binding domain which is essential for binding to the regulatory regions of target genes that contain estrogen response elements (ERE). D domain is a hinged region between the DNA binding domain (DBD) and ligand binding domain (LBD). E and F domains are the sites which involve in the binding of ligands, dimerization with another activated ER and transcriptional activation (AF-2) **(Figure 1.8).** It is important to note that the AF-1 region mediates transcription in a ligand-independent manner while transactivation by AF-2 region is dependent upon ligand binding (Matthews et al. 2003; Maggi 2011).

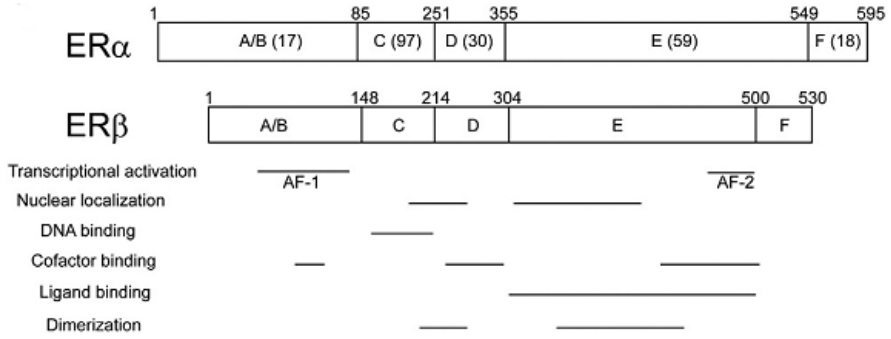


Figure 1.8: Schematic structures of estrogen receptors ERα and ERβ with their respective domains and functional regions.(Shanle et al. 2010)

1.5.2 E2 signaling via ligand-dependent genomic pathway

In the ligand-dependent genomic estrogen signaling pathway, the direct binding of estrogen to nuclear estrogen receptors initiates the response and regulate the corresponding gene expressions on long-term basis (hours to days) (Prossnitz et al. 2008; Prossnitz et al. 2008). As uncharged steroid compounds, estrogen or estrogen analogs enter freely from extracellular environment into the cytoplasmic space by the means of passive diffusion across plasma membrane and bind to ligand-binding domain on the classical estrogen receptors. These estrogen receptors present either in cytoplasm or attached on the plasma membrane. Upon ligand binding, heat-shock proteins (Hsp) that are complexed with ERs will dissociate from the ligand-bound ERs, allowing them to form homodimers and translocate into nucleus via nucleocytoplasmic shuttling. The activated ER dimers in nucleus will bind directly to estrogen responsive element (ERE), a promoter sequences of estrogen-responsive genes and act as transcription factors to recruit transcriptional co-regulators and form a preinitiation complex (Nilsson et al. 2001; Heldring et al. 2007). The binding of ERs to ERE induces the activation or repression of estrogen-regulated genes transcription, which leads to the diverse physiological effects **(Figure1.9).**

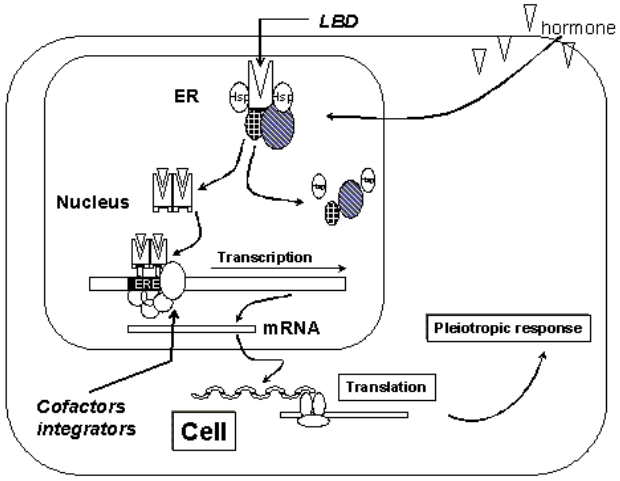


Figure 1.9: Classical ER-mediated cellular signaling pathway. (2012)

Apart from the classical ERE-directed mechanism, ERs can also indirectly modulate the expression of other target genes via alternate genomic pathways which are ligand dependent but independent of direct DNA binding **(Figure 1.10)**. This is accomplished by interactions of ERs with other proteins to form a transcription factor complex (TF) that contacts with the promoter region of target genes. It is demonstrated that the ligand-bound ERs interact with c-Fos/c-Jun dimers and other coactivators at AP-1 binding sites to regulate transcription of genes such as cyclin D1, collagenase and IGF-1 (Kushner et al. 2000; Björnström et al. 2005). A similar mechanism is also involved in the interaction of ERs with SP-1 transcription factor to induce expressions of genes containing GC-rich promoter elements (Saville et al. 2000). Another important interaction of ERs with transcriptional factors is the interaction with nuclear factor-kB (NF-kB), which leads to the repression of interleukin-6 (IL-6) expression. IL-6 is a cytokine which is responsible to trigger osteoclastic bone resorption and its expression is up-regulated by NF-kB (Tamura et al. 1993). Thus, its E2-mediated repression is believed to be pivotal in osteoporosis management (Moutsatsou 2007).

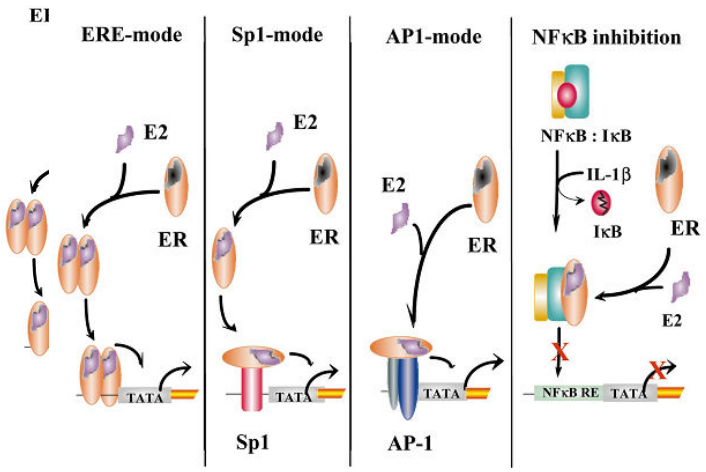


Figure 1.10: The genomic actions of estrogen receptor via classical ERE mechanism and alternate genomic pathways *(SP-1, AP-1, NF-kB)*.(Nilsson et al. 2001)

1.5.3 Activation of ER via ligand-independent phosphorylation at Ser118

Substantial studies has demonstrated that even without the presence of E2, ERα could be activated by a variety of extracellular signals, including growth factors such as epidermal growth factors (EGF) (Ignar-Trowbridge et al. 1992; Bunone et al. 1996) and insulin-like growth factor-1 (IGF-1) (Ma et al. 1994; Newton et al. 1994), cytokines and neurotransmitters such as dopamine (Smith et al. 1993). Bunone et al reported that okadaic acid, 8-bromo cAMP, EGF and dopamine effectively activated ERE-reporter gene transcription in the presence of ER, and the responses were completely abrogated by ER antagonist ICI182, 780. Furthermore, focusing on EGF, they demonstrated that mitogen-activated protein kinase (MAPK) pathway was involved in EGF-induced activation of ER (Bunone et al. 1996).

Generally, ligand-independent activation of ER is associated with activation of kinases or phosphatases involved in intracellular signaling cascades. These secondary messengers signaling pathways (MAPK, PKA, PKC, etc.) may alter the ER-dependent transcription by targeting ERs and associated proteins which lead to the phosphorylation of ERs. These actions are cell type specific, which may account for the different estrogenic responses in various cells (Coleman et al. 2001). A numbers of phosphorylation site has been mapped in ERα, comprising mostly of serine residues particularly at the AF-1 domain (Ser 104, Ser 106, Ser 118, Ser 154, Ser167, etc) and a few tyrosine residues (Tyr 52, Tyr 219, Tyr 537), each of them regulated by different upstream kinases and serve distinct biological functions (Lannigan 2003; Maggi 2011).

Among all the phosphorylation sites identified, Serine 118 residue in the AF-1 domain received much interest as it is a major phosphorylation site in response to both

estradiol binding and ligand-independent MAPK signaling (Kato et al. 1995; Bunone et al. 1996), although their kinetics and signal transduction pathways were shown to be fundamentally different (Joel et al. 1998; Chen et al. 2002). The evidence of direct action of MAPK on ERα was demonstrated by Chen et al that incubation with purified, constitutively active MAPK directly phosphorylate ERα at Ser118 without presence of E2. In addition, these studies also showed that mutation of Ser 118 to an alanine resulted in loss of activity induced by either E2 or EGF. The findings suggest that Ser118 phosphorylation plays an inevitable role in ERα function.

1.5.4 Nongenomic E2 signaling via rapid cellular signaling pathways

The classical model of estrogenic signaling suggested that the hormone has to enter the cells, bind to the ERs and induce ER translocation into the nucleus to trigger the biological responses. As mentioned, the biological responses upon hormone administration would take hours or days. However, increasing evidence showed that E2 could also rapidly induce signaling responses within seconds to minutes and the time frame is considered too short for transcriptional events to take place. To separate them with the classical "genomic" response, these rapid cellular signaling events were often described as "nongenomic" estrogenic effects.

Studies on nongenomic signaling of E2 in different cell types reported that E2 could rapidly induce a variety of secondary messenger signaling cascades. In breast cancer MCF7 cells and osteocytes, E2 could induce the rapid intracellular calcium release (Improta-Brears et al. 1999; Ren et al. 2012). E2 could also stimulate intracellular cAMP accumulation and adenylyl cyclase activity in MCF7 cells and uterine cells (Aronica et al.

1994). Besides, E2 could rapidly trigger the activation of p44/42 MAPKs (ERK1/2) in the Ras/Raf/MEK/ERK signaling cascades in osteoblasts, osteocytes, embryonic fibroblasts, endothelial cells and MCF7 cells (Migliaccio et al. 1996; Chen et al. 1999; Kousteni et al. 2001). In MCF7 cells, endothelial cells and endometrial cells, E2 also exerted its effects via p85 phosphoinositol 3-kinase (PI3K)/ Akt pathway (Ahmad et al. 1999; Castoria et al. 2001; Wei et al. 2012). These rapid activation of cellular signaling events have been attributed to the interaction of estrogenic compounds with the membrane-bound estrogen receptors, although there are still much controversies whether the nature of receptor protein involved is actually the nuclear estrogen receptors translocated to the plasma membrane or a novel G-protein estrogen receptor, GPR30 (Levin 2005; Pedram et al. 2006; Langer et al. 2010). Regardless of the nature of ER, it is clear that in response to E2, these ERs could signals through interaction with G proteins, trans-activation of growth factor receptor tyrosine kinases such as EGF receptors (EGFR) and IGF-1 receptors (IGF-1R) and other tyrosine kinases such as Src (Migliaccio et al. 2000; Prossnitz et al. 2008). This results in downstream signaling via kinases such as ERK and PI3K to phosphorylate nuclear ERs or activate transcription of target genes **(Figure 1.11).**

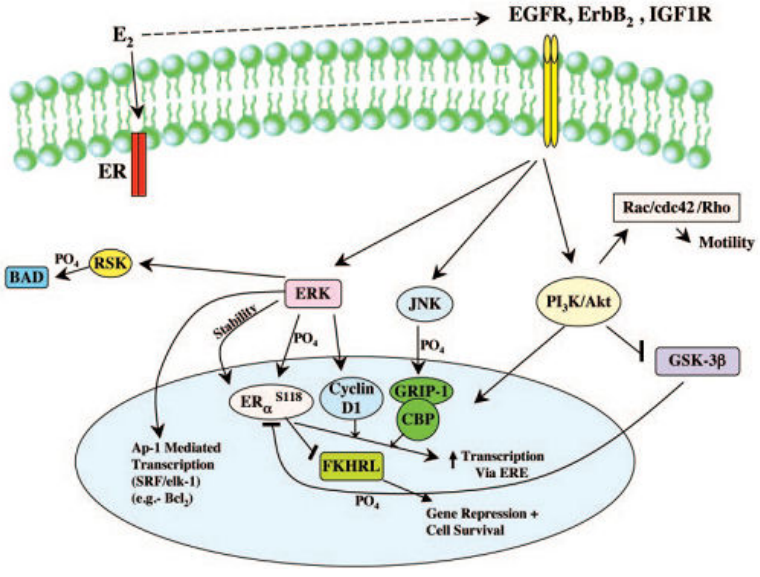


Figure 1.11: The signaling of estrogen via interaction of membrane estrogen receptor with receptor tyrosine kinases in breast cancer (Levin 2005).

MAPK Signaling Pathway

The rapid activation of the Ras/Raf/ERK pathway is perhaps one of the best characterized pathways in rapid cellular signaling of E2. Extracellular signal-regulated kinases (ERKs), also known as p44/42 MAPKs, are one of the three subgroups of MAPK together with the other two subgroups, jun N-terminal kinase (JNK) and p38 kinases (Manolagas et al. 2002). The phosphorylation and activation of the ERKs are controlled by the upstream signal transduction mechanisms. The signals from the cell surface (G-protein receptors, receptor tyrosine kinases) lead to the recruitment of accessory proteins such as Src or Shc, which would transmit the signal through various isoforms of Ras. This is followed by the Ras-induced activation of Raf, which in turn phosphorylate MAP/ERKs kinases (MEK1/2). Activation of MEK1/2 are responsible to the subsequent phosphorylation of ERKs (Coleman et al. 2001) **(Figure 1.12).** The activated MAPKs are able to modulate the estrogen-responsive gene transcription activity by at least 3 potential mechanisms: (1) via the phosphorylation of ER itself or ER-related coactivators to induce the nuclear transcriptional activities of corresponding genes; (2) via phosphorylation and activation of other transcriptional factors that interact with ER such as AP-1 or Sp-1 to act on specific sites; (3) via transactivation of genes that lack ERE-containing elements (Edwards 2005).

The activation of Ras/Raf/ERK pathway is involved in the regulation of many essential functions of the cells, including proliferation, differentiation, cell migration, survival and apoptosis, etc. These effects mediated by ERK were found to be highly tissue and cell type specific and sometimes mutually exclusive, ranging from estrogen-induced proliferation on breast carcinomas and prostate cancer cells (Migliaccio et al.

1996; Migliaccio et al. 2000) to anti-proliferative and apoptosis-inducing effects of E2 on vascular smooth muscle cells and osteoclasts (Mori-Abe et al. 2003; Chen et al. 2005).

Focusing on bone, it was reported that rapid activation of MAPK signaling by E2 might associated with increased growth and survival of osteoblasts and osteocytes, while inducing pro-apoptotic effects on osteoclasts (Kousteni et al. 2001). These bi-directional effects of MAPK signaling provide a possible mechanism by which E2 or estrogen-mimicking compounds exert their bone protective properties.

PI3K/Akt Pathway

Phosphatidylinositide 3-kinase (PI3K) exists predominantly in the form of a p85 regulatory subunit and a p110 catalytic subunit. Upon stimulation of GPCR or receptor tyrosine kinases by extracellular signals such as EGF and IGF-1, PI3K catalyzes the production of lipid mediators from their precursor phosphatidylinositol, leading to the the phosphorylation of Akt (serine/threonine protein kinase B) at the Thr308/Ser473 position and activation of endothelial nitric oxide synthase (Rameh et al. 1999). It was also demonstrated in endothelial cells and fibroblasts that ERα was able to bind PI3K in a ligand-dependent manner to activate PI3K/Akt signaling (Simoncini et al. 2000). Activation of Akt by E2 or IGF-1 plays a significant role in promoting cell proliferation and preventing cell apoptosis (Ahmad et al. 1999). In vascular endothelial cells, E2-induced phosphorylation of Akt occurred as rapid as 5 minutes, peaked at 15 minutes and lasted for 6 hours. The DNA microarray results showed that E2, via PI3K activation, was able to induce up-regulation of 250 genes that could be abolished by PI3K inhibitor LY294002 (Pedram et al. 2002). These genes include c-fos, c-jun in the AP-1 complex

which is essential for cell cycle progression and proliferation, and genes involved in osteogenic functions, including bone morphogenetic protein 2 (BMP2), osteoblast specific cysteine rich protein and AREB6 transcription factor which are associated with osteoblastic functions and differentiation of osteoblast-precursor cells.

The importance of PI3K/Akt signaling in bone homeostasis could be seen by the osteopenic phenotype of Akt1 knockout mice due to dysfunction of both osteoblasts and osteoclasts (Kawamura et al. 2007). Akt is also found to be positively correlated with the anabolic activity, differentiation and cell survival of osteoblasts (Dufour et al. 2007; Liu et al. 2007). In the study, we sought to explore the possibilities of our flavonoid compounds to exert their effects via PI3K/Akt signaling in osteoblasts.

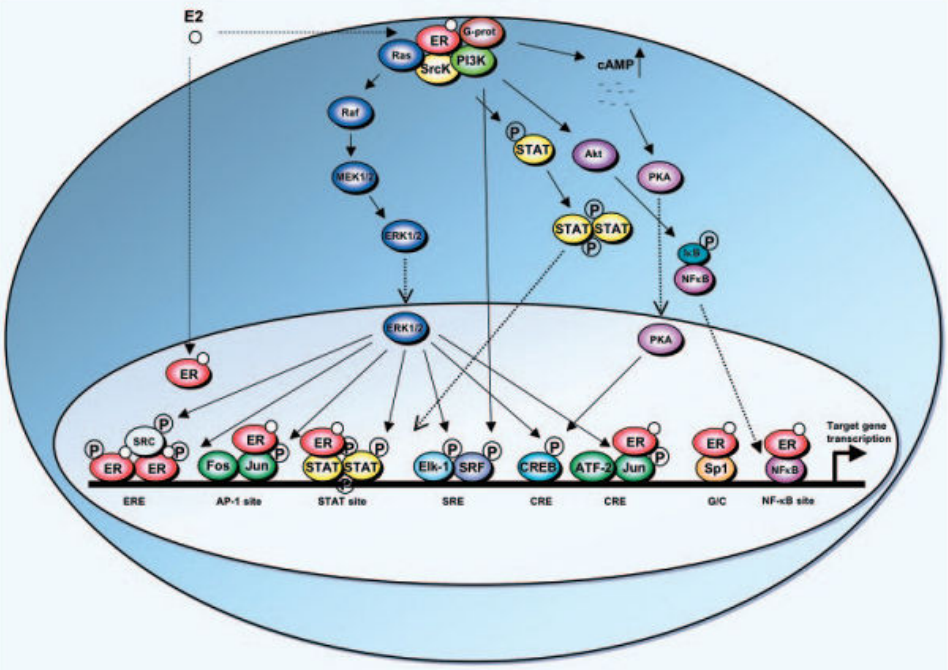


Figure 1.12: Model of nongenomic actions of ERs via rapid cellular mechanisms, including Ras/Raf/MEK/ERK pathway, PI3K/ Akt pathway and cAMP/PKA-mediated signaling (Björnström et al. 2005).

1.5.5 GPR30 as an estrogen receptor

Being the largest family of integral membrane receptors involved in cellular signaling, G protein-coupled receptors (GPCRs) are well known to play indispensable roles in the regulation of individual's physiology, growth, development and disease control by mediating the intracellular biochemical signal transmission(Stadel et al. 1997; Marinissen et al. 2001; New et al. 2007). The general structure which defines a GPCR consists of an extracellular N-terminal segment, 7 transmembrane (TM) domain, 6 loops (3 extracellular, 3 cytoplasm) and an intracellular C-terminal segment (Figure 1.3). They are activated in response to a wide variety of ligands from extracellular environment such as neurotransmitters, lipids, hormones, peptides, proteins, growth factors, light, and transmit the signals into cytoplasm **(Figure 1.13)**.

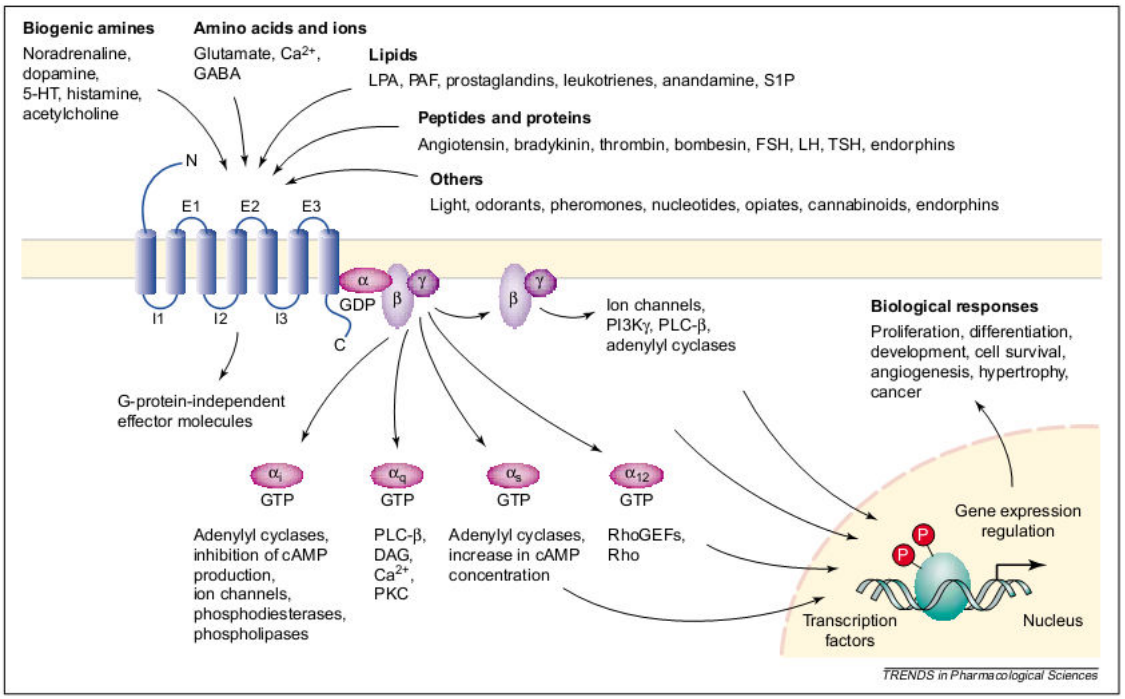


Figure 1.13:Signaling pathway triggered by activation of GPCR (Marinissen et al. 2001).

Since the discovery and intensive studies on the classical estrogen receptors ERα and ERβ, it was thought that the actions of these two classical ERs are sufficient to cover most, if not all, of the functions of estrogen (Prossnitz et al. 2009; Maggiolini et al. 2010), and that there will be no "need" for the involvement of other receptors to play major roles in mediating estrogenic effects. This idea, however, was challenged by the fact that estrogen can also trigger signaling cascades that occur more rapidly than transcriptional events attributed to ER and that these events could not be abrogated by classical ER inhibitors, leading to the speculation that estrogen may signal via other receptors as well (Filardo et al. 2002). That is why the recent identification of a 7 transmembrane G protein-coupled receptor, GPR30 as a potential estrogen receptor gained much attention in the research on estrogen signaling.

GPR30, or recently named GPER1 (G protein-coupled estrogen receptor 1), was first cloned in parallel by multiple laboratories groups between 1996 and 1998 (Bonini et al. 1997; Carmeci et al. 1997; Feng et al. 1997) using distinct methodologies. As it showed low homology to other GPCRs and the ligand for it was unknown at that time, it was identified to be an orphan 7 transmembrane GPCR, designated GPR30. It was not until 2000 that the association between estrogen and GPR30 was discovered.

In 2000, study by Filardo and colleagues in breast cancer cell lines identified GPR30 as a nonclassical membrane-bound receptor that showed interaction with estrogen (Filardo et al. 2000). Estrogen was found to activate Erk-1/-2 in MCF-7 cells which express both ERs and GPR30 ($ER\alpha^+$, $ER\beta^+$, $GPR30^+$) and also in SkBr3 breast cancer cells which only express GPR30 ($ER\alpha^-$, $ER\beta^-$, $GPR30^+$), but fail to induce Erk-1/-2

activation in MDA-MB-231 cells which are GPR30 deficient ($ER\alpha^-$, $ER\beta^+$, $GPR30^-$). Transfection of GPR30 into MDA-MB-231 cells restored this estrogen-induced response. These findings suggest that GPR30 is responsible for estrogen-dependent nongenomic rapid signaling. In 2005, studies by two independent groups had demonstrated the direct binding of estrogen to GPR30 using fluorescent estrogen analog (Revankar et al. 2005) and radiolabelled E2 binding assay(Thomas et al. 2005). Although the two groups have discrepancy in whether GPR30 localizes in plasma membrane or endoplasmic reticulum, they both provided strong evidence that GPR30 acts as a membrane-bound estrogen receptor which interacts with estrogen via direct binding.

GPR30 in Bone

Using immunodetection method, the GPR30 protein expression was recently identified in human bone (Chagin et al. 2007). The study demonstrated that GPR30 was expressed in the cartilage of human epiphyseal growth plate. The highest level of GPR30 expression was detected in the terminally differentiated chondrocytes of hypertrophic and resting zone rather than the proliferative zone, suggesting that GPR30 may regulate chondrogenesis in the growth plate. Subsequent study by the same group (Heino et al. 2008) focusing on bone cells demonstrated that GPR30 expression was detected in all three bone cell types: osteoclasts, osteoblasts and osteocytes **(Figure 1.14)**. Osteocytes generally possesses more GPR30 than osteoblasts. Together with the result of previous study, it was suggested that GPR30 expression is regulated by cell differentiation and generally higher in the more differentiated state of bone formation cells. Besides, study in rat calvarial preosteoblasts demonstrated that Runx2, an inducer of osteoblast differentiation, was able to increase GPR30 gene expression, which in turn increased

proliferation of the preosteoblasts cells (Teplyuk et al. 2008). These results provide a strong scientific basis that GPR30 has a role in bone metabolism.

The importance of estrogen has long been known in controlling the normal bone growth and maintaining the balance on bone homeostasis. Studies of estrogenic responses on bone clearly demonstrate that estrogen treatment increases trabecular and cortical bone mass, promotes bone mineral density(BMD), inhibits longitudinal bone growth and promotes growth plate fusion by reducing width of growth plate(Olde et al. 2009; Windahl et al. 2009). In vivo study by Martesson et al first reported that female $GPR30^{-/-}$ mice exhibited reduced skeletal growth measured by a minor decrease in crown-rump and femur length (Mårtensson et al. 2009). Subsequent study by Windahl et al demonstrated that E2-mediated regulation of longitudinal bone growth and growth plate height was absent in female $GPR30^{-/-}$ mice, suggesting the function of GPR30 is required for these estrogenic effects in bone (Windahl et al. 2009). However, E2 responses in several bone mass parameters including total body BMD and cortical thickness showed no significant difference between wild type and $GPR^{-/-}$ mice. Thus, the authors conclude that GPR30 is required for normal estrogenic response in regulating the growth plate rather than for the direct osteoprotective effect of estrogen. As there are still lack of information on the skeletal studies on GPR30 knockout mice models by date, the functions and role of GPR30 in regulating the bone metabolism are yet to be determined and require further studies.

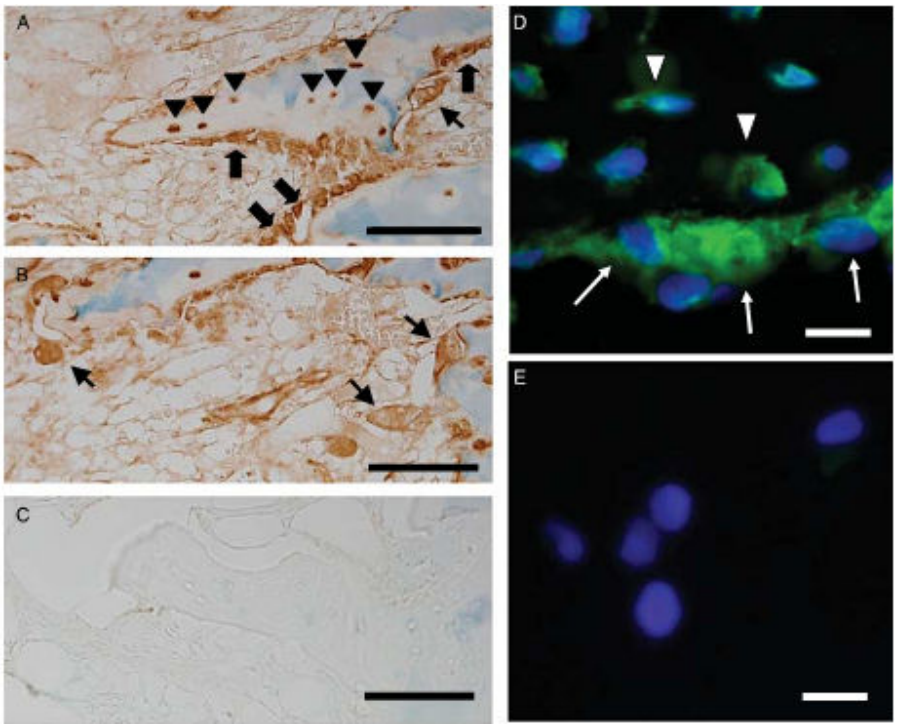


Figure 1.14: GPR30 expression in human bone cells (Heino et al. 2008).

(A) Staining of GPR30 in osteocytes (arrowheads), osteoblasts (block arrows) and osteoclasts (arrows). (B) GPR30 staining in osteoclasts (arrows). (C) Negative control. (D) Cytoplasm staining of GPR30 in osteoblasts (arrows) and osteocytes (arrowheads). (E) Negative control.

1.5.6 E2 signaling via GPR30-mediated pathway

Recent studies revealed that E2 or the estrogen-like compounds are able to regulate the long-term genomic effects via direct binding and activation of classical estrogen receptors (ERs), and also via the rapid cellular signaling cascaudes through nongenomic pathway through the activation of cell surface receptors such as membrane-bound ERs and G protein-coupled Estrogen Receptor GPR30.

The model of nuclear ER-mediated mechanism provides a direct explanation on how the target cells respond to E2 signaling. However, it was later discovered that estrogenic compound did not act solely on classical ERs and much complex signaling pathways might be involved (Genazzani 2006; Zhang et al. 2008).This includes the ability of E2 to activate several kinase-mediated signaling cascades including extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways, both requiring the up-stream activation of cell surface receptor instead of nuclear localization of the activated ER (Kousteni et al. 2001).

By date, our understanding of GPR30-mediated estrogenic signaling relies mainly on in vitro studies in breast cancer cell lines (Filardo et al. 2000; Filardo et al. 2002). These studies have demonstrated that the membrane bound GPR30 receptor binds specifically with estrogen and their subsequent studies reported the molecular mechanism involves in the signal transduction of GPR30 in response to estrogen. It was found that GPR30 interacts with estrogen to activate its corresponding heterotrimeric G protein, which in turn release the α subunit from the β and γ subunits. The dissociated α subunit induces the intracellular production of cAMP via activation of adenylyl cyclase (AC), while β andγ subunits activate Src-like tyrosine kinase and facilitate its interaction with

adaptor protein Shc to form a complex. The latter pathway is found to be involved in activation of matrix metalloproteinase (MMP) which in turn act on pro-HB-EGF to release heparin-binding EGF-like growth factor (HB-EGF) into extracellular space. HB-EGF then transactivate EGF receptors (EGFR), which leads to various downstream signaling cascades that involve MAP Kinase/ERK-mediated pathways, PLC activation, and PI3K-related pathways. The activation of these signaling cascades not only initiates rapid cellular responses such as calcium mobilization, ion channels activation and intracellular cAMP accumulation, but also results in the activation or recruitment of various transcriptional factors and proteins (Edwards 2005) **(Figure 1.15).** The effect of these GPR30-mediated responses to E2 leads to the proliferation of breast cancer cells.

However, recent studies suggested that besides the rapid cellular responses that lead to short-term effects, GPR30 has the capability to regulate transcriptional activities as well (Kang et al. 2009). This is due to the fact that the binding of ligand to GPR30 activates mitogen-activated protein kinases (MAPKs) via downstream signaling cascades. Considering the possible cross-talk between the genomic pathway and nongenomic signaling and possible overlapping ligand-binding profiles of both the classical ERs and GPR30, the estrogenic compounds may exert their effects via the activation and interaction of ER and GPR30-dependent pathways.

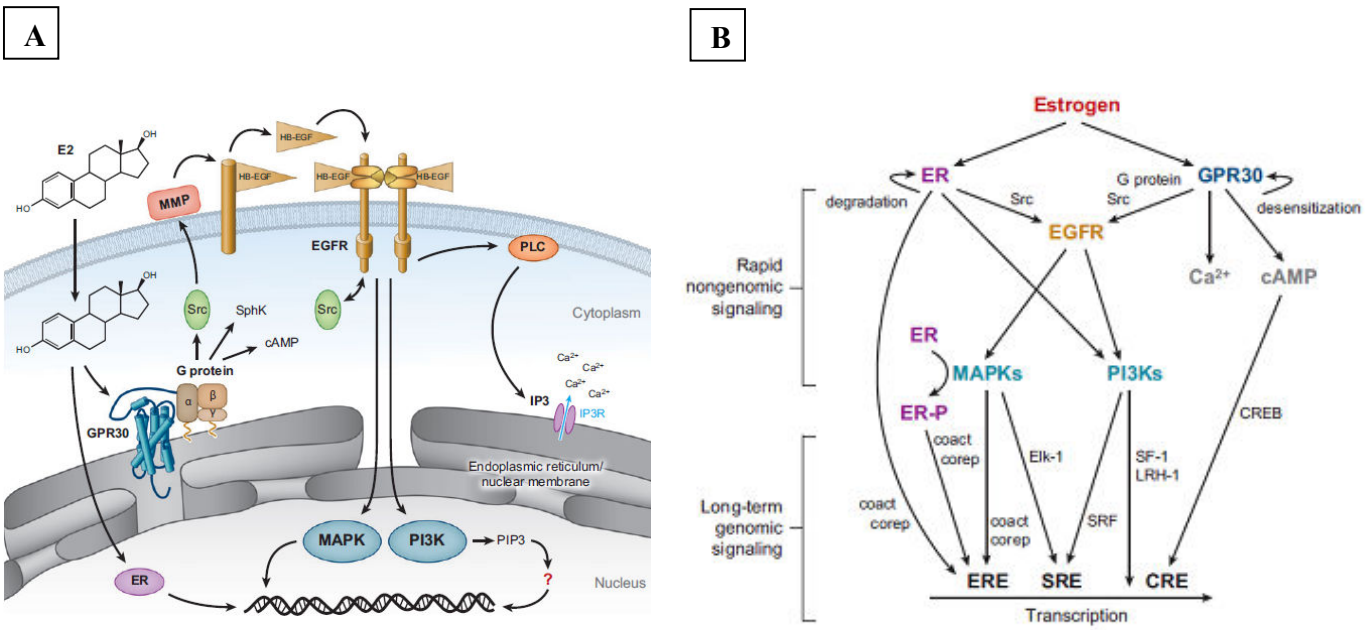


Figure 1.15: Model in comparison of classical ER-mediated genomic pathway and GPR30-mediated nongenomic pathway(Prossnitz et al. 2008)

(A) Estrogen could exert its effects via direct binding to the classical ERs in the cytoplasm to induce genomic signaling. On the other hand, E2 could also bind and activate the cell surface GPR30 to induce a series of downstream signaling cascades which involves molecules such as cyclic AMP(cAMP) production, MAPK and PI3K signaling which could regulate the transcription factors as well.

(B) The proposed mechanism of ER-mediated pathway and GPR30-mediated pathway and the possible cross-linkage between these two signaling systems upon binding of estrogen. ERE: estrogen response element, SRE: serum response element, CRE: cAMP response element.

1.5.7 ER and GPR30 antagonists

ICI182, 780 (ICI), commonly known as fulvestrant, is a specific ER antagonist that is widely used for antagonizing the interaction of estrogenic compounds with classical ER (Kansra et al. 2005; Mok et al. 2010; Pang et al. 2010). Pre-treatment of the cells with ICI could effectively down-regulate the protein expression of ER and disrupts the nucleocytoplasmic shuttling by preventing the transfer of activated ER into the nucleus (Dauvois et al. 1993; Kansra et al. 2005)

On the other hand, G15 is a compound recently characterized and identified to exert its anti-estrogenic effect via selective inhibition on GPR30. The ligand-binding studies found that G15 demonstrated a high binding affinity and specificity to GPR30 while showing little to no binding towards classical ERα and ERβ. It is able to antagonize GPR30-mediated PI3K activation and intracellular calcium mobilization by estrogen in vitro using breast cancer model (Dennis et al. 2009; Jenei‐Lanzl et al. 2010) Hence, pre-treatment with G15 would be assumed to antagonize the effects of the phytoestrogens that require the activation of GPR30-mediated cellular signaling. However, the antagonizing effects of G15 on estrogenic actions in osteoblasts are still scarcely investigated by date.

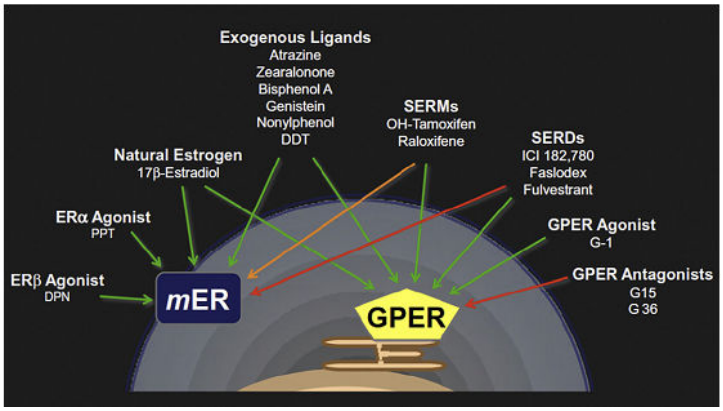


Figure 1.16: Agonists and Antagonists of membrane ER and GPER (Barton 2012)

Chapter 2

Hypothesis
And
Objectives

2.1 Hypothesis

Our previous findings on Naringin, Icariin and Genistein demonstrated that they exerted estrogen-like effects on bone metabolism. However, for naringin and icariin, we have shown that their actions in osteoblasts were estrogen receptor dependent but independent of ERE-mediated genomic transcription. Thus, we hypothesized that the flavonoids exert their estrogen-like effects on osteoblasts without direct binding to the ERs via non-genomic rapid cellular signaling system to induce ligand-independent activation of ER pathway.

Recently, it had been found that estrogen exerted its effects not only via the activation of classical estrogen receptors, but also via the activation of a novel membrane bound estrogen receptor, GPR30. In light of this, we also hypothesized that the activation of GPR30 might involve in mediating the non-genomic estrogenic signaling exerted by the flavonoids in osteoblastic cells.

2.2 Objectives

This study aims to elucidate the involvement and molecular mechanisms of GPR30-mediated rapid cellular signaling in phytoestrogen-induced anabolic effects in rat osteoblastic UMR106 cells. The objectives are:

(1) To determine if naringin, icariin and genistein exert their anabolic effects in UMR106 cells and if their actions are dependent on the classical estrogen receptors (ERs) and the novel G-protein estrogen receptor (GPR30) in osteoblasts

(2) To determine the molecular mechanisms of the phytoestrogens in estrogenic signaling via classical genomic pathway and/or rapid non-genomic estrogenic signaling in osteoblasts.

(3) To delineate the possible correlation between rapid cellular signaling of ER by phytoestrogens with GPR30-mediated non-genomic signaling in osteoblasts.

Chapter 3

Involvement of ER and GPR30-mediated signaling in phytoestrogens-induced anabolic effects on osteoblasts

3.1 Introduction

It is well established that estrogen administration could effectively prevent postmenopausal bone loss not only by inhibiting resorption activity of osteoclasts, but also by exerting anabolic effects on bone formation as well. Previous studies of our group clearly suggested that naringin (Pang et al. 2010), icariin (Mok et al. 2010) and genistein (Chen et al. 2006) at various concentrations improve bone properties by exerting estrogen-like anabolic effect on both in vivo and in vitro model. Interestingly, in these studies, naringin and icariin were found to be acting on classical ERs in a ligand-independent manner without inducing ERE-mediated transcription, meaning that it was not necessary for the direct binding of these phytoestrogens to the classical ERs to exert their effects. It also suggested that an alternative estrogenic signaling route might be utilized by these flavonoid glycosides which involved proteins other than classical ERs to exert their anabolic effects.

Activation of the novel G protein estrogen receptor (GPR30) by ligand binding had been shown to induce estrogenic responses in vitro such as stimulating cell proliferation in certain cell types (endometrial, ovarian cancer cells) but reducing proliferation in others (bladder urothelial cells) (Chan et al. 2010). This suggests that the role of GPR30 in mediating cell growth is cell type specific, and the biological importance of GPR30 in cells of osteoblastic lineage is yet to be discovered. In this study, we explored the possibilities of GPR30 involvement in the estrogenic effects of the phytoestrogens in osteoblasts.

UMR106 cells were utilized as the in vitro model in this study. UMR106 cell is an ^{32}P-induced transplantable osteosarcoma derived from Sprague-Dawley rat which

expresses osteogenic phenotypes (Partridge et al. 1983). It was reported that this cell line is high in alkaline phosphatase (ALP) activity, abundant in receptors for hormones (VDR, PTH receptor) and cell surface receptors such as IGFR, EGFR etc. (Mitchell et al. 1990; Armbrecht et al. 1998; Gruber et al. 1999), making it a useful model for studying hormonal regulation of cells with osteoblastic lineage. The expression of estrogen receptors ERα and ERβ were also detected in UMR106 cells, despite the fact that their expression levels were found to be considerably low (Davis et al. 1994). It has also been used routinely as a relatively developed osteoblast model system for investigating the effects of various anti-osteoporotic treatments including parathyroid hormones (PTH) and estrogenic compounds on osteoblasts *in vitro* due to its well characterized estrogenic responses typical of an osteoblast (Qin et al. 2003). Its stability in retaining osteoblastic characteristics in each passage is also an advantage for investigation on cellular signaling events at the molecular level.

One of the important estrogenic responses in osteoblasts is the increase in alkaline phosphatase (ALP) activity. ALP plays a pivotal role in the calcification of bone. ALP activity is associated with the production of inorganic phosphate required for bone mineralization and the hydrolysis of substances that inhibit calcification such as extracellular pyrophosphate under alkaline condition (Golub et al. 2007; Lee et al. 2007);. The elevation in the ALP activity level in osteoblasts is widely regarded as a marker indicating active bone formation, differentiation and calcification.

In the current study, the potential anabolic effects of naringin, icariin and genistein at a wide range of concentrations on UMR106 cells were examined. Their abilities to enhance cell proliferation and differentiation were assessed by MTS Assay and ALP assay,

respectively. ER and GPR30 antagonists assay on cell proliferation and differentiation were performed in which the rat osteoblastic UMR106 cells were undergone pre-treatment of ER antagonist (ICI182780) or GPR30 antagonist (G15) before treatment with the compounds. These assay aimed to elucidate the dependency of phytoestrogens on these 2 receptors to exert their estrogenic effects in promoting osteoblastic cell proliferation and differentiation.

Our previous studies had also indicated that both naringin and icariin, at various concentrations tested, would increase the mRNA expression of OPG and the OPG/RANKL ratio in UMR cells (Mok et al. 2010; Pang et al. 2010). Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of tumor necrosis factor ligand family, plays a significant role in osteoclast formation. Osteoprotegrin (OPG) is a decoy receptor produced by osteoblasts which binds to RANKL, thereby inhibiting the availability of RANKL to bind to its functional receptor, RANK to induce osteoclastogenesis (Khosla 2001). The increase of ratio between the expression levels of OPG and RANKL indicates the ability of the particular flavonoid in modulating osteoclastogenesis. While various studies have found that ICI182780, the ER antagonist, is able to abolish the effect of 17β-estradiol and phytoestrogens in modulating OPG/RANKL in osteoblasts (Bord et al. 2003; Mok et al. 2010; Pang et al. 2010), it is still unknown that pre-treatment of E2 or phytoestrogens with G15 would lead to any antagonizing effect in this aspect.

In this study, the role of GPR30 activation in the effect of estrogen and phytoestrogens in modulating osteoclastogenesis was investigated. The basal mRNA expression of GPR30 in UMR106 cells was detected by PCR followed by gel

electrophoresis. The most potent concentrations of naringin, icariin and genistein in inducing cell proliferation and differentiation, along with E2 and vehicle, were used in treatment with or without G15 in UMR106 cells and their effects on mRNA expression of OPG and RANKL were measured by real time RT-PCR analysis.

3.2 Methodology:

3.2.1 Cell Culture

Rat osteoblastic UMR106 cells: UMR 106 cells (ATCC® Number: CRL-1661™**)** were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) which is supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics P/S (100U/mL Penicillin, 100ug/mL Streptomycin, Gibco). The cells were cultured on 100mm culture dishes and incubated in a 37°C incubator which provides the atmosphere of 95% air humidity and 5% CO_2. Cells were harvested with trypsin (0.05% Trypsin-EDTA, Gibco) and sub-cultured when they had reached approximately 80~90% confluence in the plate.

Seeding of cell for drug treatment

The UMR106 cells were seeded at different densities depending on the requirement of different assays (Table 3.1). After 48 hours incubation, the culture medium was replaced by phenol red-free DMEM supplemented with 1% dextran-charcoal-stripped-serum (sFBS, Gibco) and P/S to provide a sterol-starved condition. Drug treatment commenced after incubation in the sterol-starved medium for 24 hours.

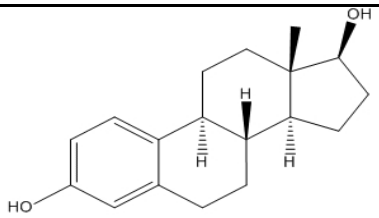
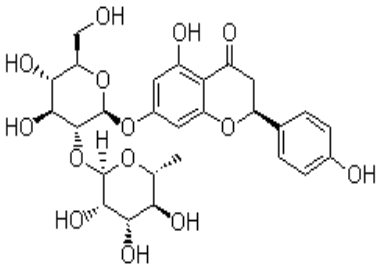
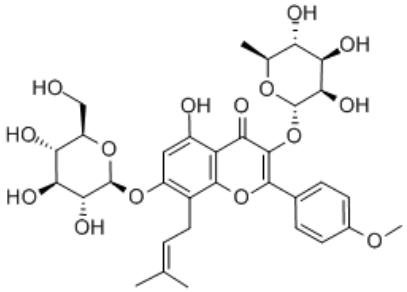
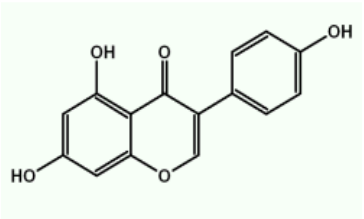
Table 3.1: List of different UMR106 cell densities seeded for different assays.

	6-well plate	24-well plate	96-well plate
Cell concentration required(cells/ml)	**100,000**	**70,000**	**55,000**
Volume/well	**2ml**	**500ul**	**100ul**
Assays	**Immunoblotting, real time RT-PCR, cAMP EIA assay**	**Dual luciferase activity assay, immunoblotting**	**MTS assay, ALP assay**

3.2.2 Drug Preparation and Treatment

17β-estradiol (#E8875, purity ≥ 98%), Naringin (#N1376, purity ≥ 90%) and Genistein (#G6649, purity ≥ 98%) were purchased from Sigma-Aldrich (St Louis, MO, USA), while Icariin (#I0901, purity ≥ 98%) was purchased from LKT Laboratories Inc (St Paul, MN, USA). All of the stocks came in powder form. The drugs were dissolved in their solvent respectively as mentioned in Table 3.2 to form 10^{-2}M stock solution and subsequent dilution was performed using absolute ethanol into a lower desired dosages. The concentrations of different phytoestrogens used for treatment ranged from 10^{-12}M to 10^{-5}M, which were determined based on the reference of previous studies. 17β-estradiol (E2, 10^{-8}M) was used in this study as positive control group.

Table 3.2: Profiles of 17β-estradiol and flavonoids in the study

Compound	Chemical formula	Chemical structure	Molecular Weight(g/mol)	Solubility
17β-estradiol	$C_{18}H_{24}O_2$		272.38	Absolute ethanol
Naringin	$C_{27}H_{32}O_{14}$		580.54	Absolute ethanol
Icariin	$C_{33}H_{40}O_{15}$		676.662	DMSO, Diluted with absolute ethanol
Genistein	$C_{15}H_{10}O_5$		270.24	Absolute ethanol

*All data and figures were obtained from Wikipedia(www.wikipedia.org)

3.2.3 Cell Proliferation Assay

The CellTiter 96®AQueous cell proliferation assay (Promega) was a colorimetric assay to determine the amount of viable cells in the culture. Briefly, the mitrochondrial dehydrogenases in metabolically active cells are able to reduce the chromogenic MTS substrate into a soluble, brownish formazan compound **(Figure 3.1)** with the addition of the electron coupling reagent phenazine methosulfate (PMS). The resulting product could be detected directly by measuring the absorbance at 490nm, and the results serve as a resemblance to the viable cells amount in the culture.

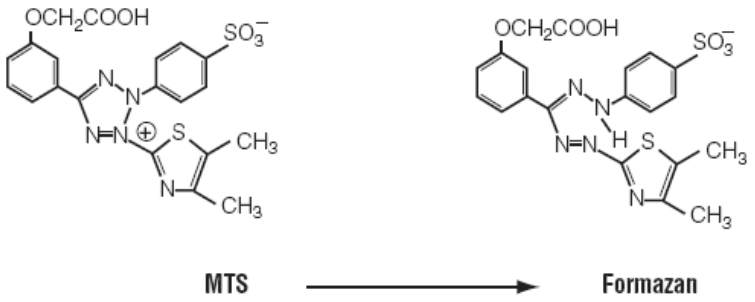
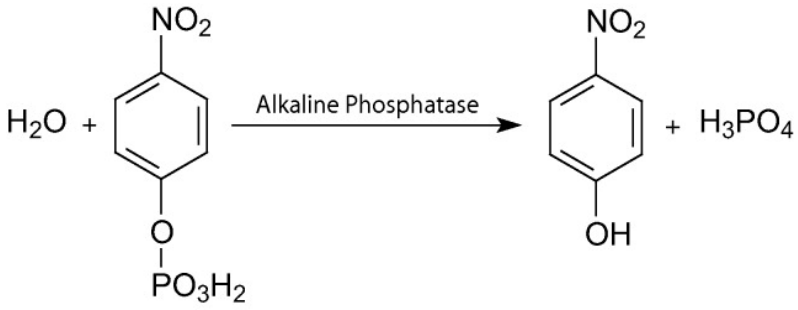


Figure 3.1: The conversion of MTS to formazan product by dehydrogenase

MTS assay were performed after 48 hours of treatment with the compounds. The MTS assay solution was prepared as described in the manual and 120μL of the MTS solution were added into each sample well. The 96-well plate was then incubated for an hour at the 37°C incubator. The absorbance of each well was measured at 490nm using a microplate-reader (Bio-Rad, Model 550) and the results were expressed as cell proliferation relative to control (OD_{490nm} of sample/OD_{490nm} of control).

3.2.4 Cell Differentiation Assay

Cell differentiation of UMR106 cells were assessed by measuring the activity of alkaline phosphatase (ALP), a marker for osteogenic differentiation. The assay utilized p-nitrophenyl phosphate (p-NPP, Sigma-Aldrich) as the substrate for ALP. Alkaline phosphatase hydrolyze p-NPP to the yellowish p-nitrophenol by removing its phosphate group **(Figure 3.2)**, and the concentration of the product was measured by the absorbance at 405nm.



p-nitrophenylphosphate **p-nitrophenol**

Figure 3.2: Action on p-NPP by alkaline phosphatase.

The cells were seeded in 96-well plate and treated with the compounds for 24 hours. After treatment, the cell were lysed with passive lysis buffer (PLB, Promega) and incubated at -80°C for 10-15 minutes followed by thawing the plate at room temperature. The freeze-and-thaw cycle enabled the break up of the cells and inactivation of proteolytic enzymes. 10mM p-NPP dissolved in PBS was then added to each well, followed by a constant shaking in a hybridizer at 37°C for 30 minutes. The absorbance at 405nm was measured using the 96-well microplate reader.

The results of the ALP assay were normalized with total protein content in the cells by Bradford protein assay. The cells were lysed with PLB and Protein Assay Dye

Reagent Concentrate (BioRad, #500-0006) was added into the wells followed by incubation for 10 minutes at room temperature. Absorbance at 595nm was measured in a microplate reader.

ALP activity of sample after normalization was expressed as $OD_{405nm}$of sample/OD_{595nm} of sample.

3.2.5 Reverse transcription-Real time PCR

Total RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction

UMR106 cells were seeded in 6-well plate and treated with compounds for 48 hours. Trizol reagent (Life Technologies) was used to extract the total RNA from the cells following the standard protocol. The purity and RNA concentration of the samples were measured and 2μg of total RNA was used to generate cDNA strands using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following the manual. The cDNA products of RT-PCR were stored at -20°C and were used as template to perform real-time quantitative polymerase chain reaction.

Real-time Polymerase Chain Reaction and Gel Electrophoresis

The cDNA products from RT-PCR served as the template for the subsequent real-time quantitative PCR using the primers as listed in Table 3.3.

Table 3.3: Sequences of primers

Gene	Alignment	Sequence	Tm (°C)	Primers concentration (uM)	Product length (bp)
OPG	Forward Reverse	GTTCTTGCACAGCTTCACCA AAACAGCCCAGTGACCATTC	55	0.2 0.2	121
RANKL	Forward Reverse	CATCGGGTTCCCATAAAGTC CTGAAGCAAATGTTGGCGTA	55	0.2 0.2	142
GPR30	Forward Reverse	TCTTCATCAGCGTCCACCTAC TTGTCCCTGAAGGTCTCTCC	56	0.2 0.2	172
GAPDH	Forward Reverse	TGGCATCGTGGAAGGGCTCAT CCACCACCCTGTTGCTGTAAC	60	0.2 0.2	473

Real time quantitative PCR reaction was performed using the iCycler with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The results in the amplification chart (Related Fluorescent Unit, RFU vs cycle) were used to determine the mRNA expression of RANKL, OPG, GPR30 and GAPDH. The purpose of including GAPDH, a housekeeping gene is to normalize the expression levels of RANKL, OPG and GPR30 genes. A standard curve was plotted to determine the copy number of cDNA template relative to control. Post-PCR melting curves are used to analyze the specificity of the amplification.

The molecular sizes of the PCR products were analyzed using DNA gel electrophoresis. The DNA bands were visualized by ChemiDoc™ XRS (Bio-Rad) imaging system and the length of the qPCR products were analyzed and compared to the expected length of PCR amplification of GPR30 gene.

3.2.6 ER and GPR30 antagonist assay

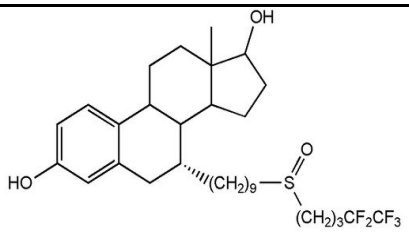
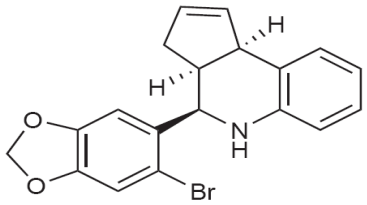
	Chemical formula	Chemical structure	Molecular Weight(g/mol)	Solubility
ICI 182780	$C_{32}H_{47}F_5O_3S$		606.772	DMSO (100mM), absolute ethanol (50mM)
G15	$C_{19}H_{16}BrNO_2$		370.2	DMSO

Table 3.4: Chemical profiles of ICI182780 and G15

For the antagonist study, the cells were pre-treated with ER antagonist ICI182780 (ICI, 10^{-6}M) or GPR30 antagonist G15 (10^{-6} M) for 20 minutes prior to drug treatment to effectively antagonize the possible activation of the classical ERs and GPR30 receptor. The optimum doses of the flavonoid compounds that induced the greatest stimulatory effect in the proliferation and differentiation of UMR106 cells was used in the antagonist study based on the findings on the previous assays. The subsequent steps were performed according to procedures as described in each assay and the results for the drug treatment in the absence or presence of antagonists were compared.

3.2.7 Statistical Analysis

All results were expressed as mean ±standard error of mean (SEM) and were analyzed by two-tailed unpaired Student's t-test between the drug-treated group and control group using Microsoft Office Excel 2007.A value of $p < 0.05$ was considered significant. All graphs in the study were plotted using GraphPad Prism Version 4.00.

3.3 Results

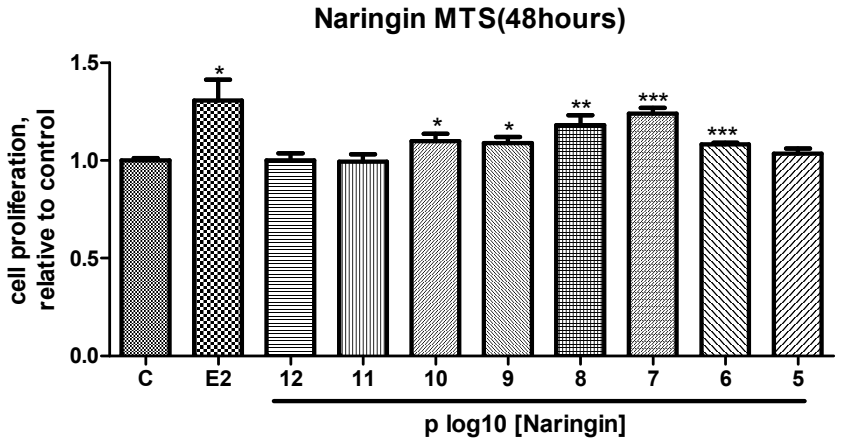
3.3.1 Characterization of anabolic effects of flavonoids in cell proliferation and differentiation of osteoblasts

The effects of flavonoids at various concentrations on UMR106 cell proliferation and differentiation were examined after treatment for 48 hours and 24 hours, respectively. 17β-estradiol (E2, 10^{-8}M) was found to significantly increase the cell proliferation for at least 1.2-fold ($P<0.05$) upon incubation for 48 hours. ALP activity were significantly stimulated for at least 1.23-fold ($P<0.01$ vs vehicle) when treated with 17β-estradiol (E2, 10^{-8}M) for 24 hours.

Naringin

Treatment of UMR106 cells with naringin (10^{-7}M to 10^{-10}M) for 48 hours and 24 hours effectively increased the osteoblastic cell proliferation and differentiation, respectively. The concentration which demonstrated most potent effect in stimulating cell proliferation and differentiation were found to be 10^{-7}M. The cell growth of UMR 106 cells was significantly increased by around 1.25-fold upon treatment with 10^{-7}M naringin ($P<0.001$) and around 1.18-fold upon treatment with 10^{-8}M naringin ($P<0.01$). ALP activity of the UMR 106 cells was significantly increased by around 1.36-fold upon treatment with 10^{-7}M naringin ($P<0.001$) and around 1.18-fold upon treatment with 10^{-8}M naringin ($P<0.01$) Lower concentrations of naringin (10^{-9}, 10^{-10}M) also demonstrated significant stimulatory effect in promoting cell proliferation as well as differentiation in UMR106 cells ($P<0.05$), while no significant difference were observed when cells were treated with either higher dosage (10^{-5}M) or the lower doses (10^{-11}, 10^{-12}M).

A



B

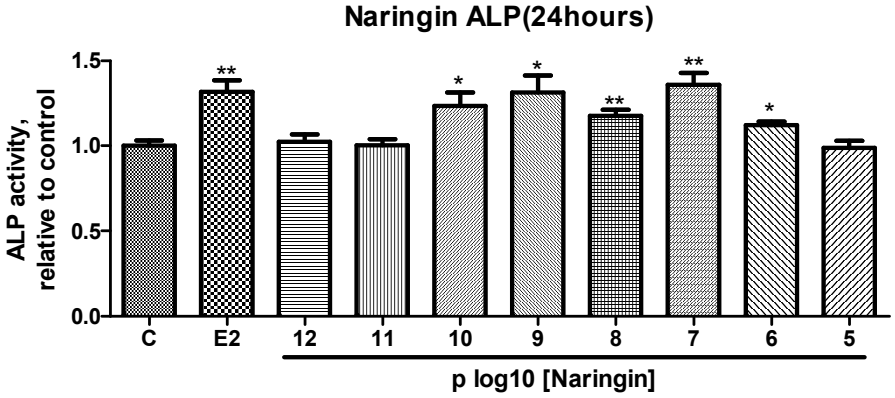


Figure 3.3: Effects of treatment with naringin on UMR106 cell proliferation and ALP activity.

5,500 cells/ well were cultured in 96-well microtiter plate and were subjected to drug treatments for 24/48 hours. **(A)** Cell proliferation rate was assessed by MTS assay. **(B)** Cell differentiation was indicated by ALP activity in the assay normalized with total protein content (OD_{595}) in Bradford assay. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M) or Naringin (10^{-12}~10^{-5}M). Results were obtained from two experiments (n=6) and expressed as mean ± SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

Icariin

Results showed that icariin was potent in promoting both the osteoblastic proliferation and differentiation. Culture of icariin-treated UMR106 cells at higher concentrations, i.e.10^{-9}M to 10^{-6}M, for 48 hours significantly promoted cell proliferation by around 1.2-fold ($P<0.01$, vs vehicle). The proliferative effects of icariin were showed to be dependent on its concentration as the potency of lower concentrations (10^{-12}M to 10^{-10}M) was not as apparent as the higher concentrations.

In contrast, icariin showed more potency in stimulating ALP activity at a wider range of concentrations, particularly at the lower concentrations such as 10^{-12}M and 10^{-10}M. At the concentration of 10^{-12}M, ALP activity in UMR106 cells was increased by 1.36-fold ($P<0.001$ vs vehicle). The effect of icariin on promoting cell differentiation remained significant at all concentrations in 24 hours treatment.

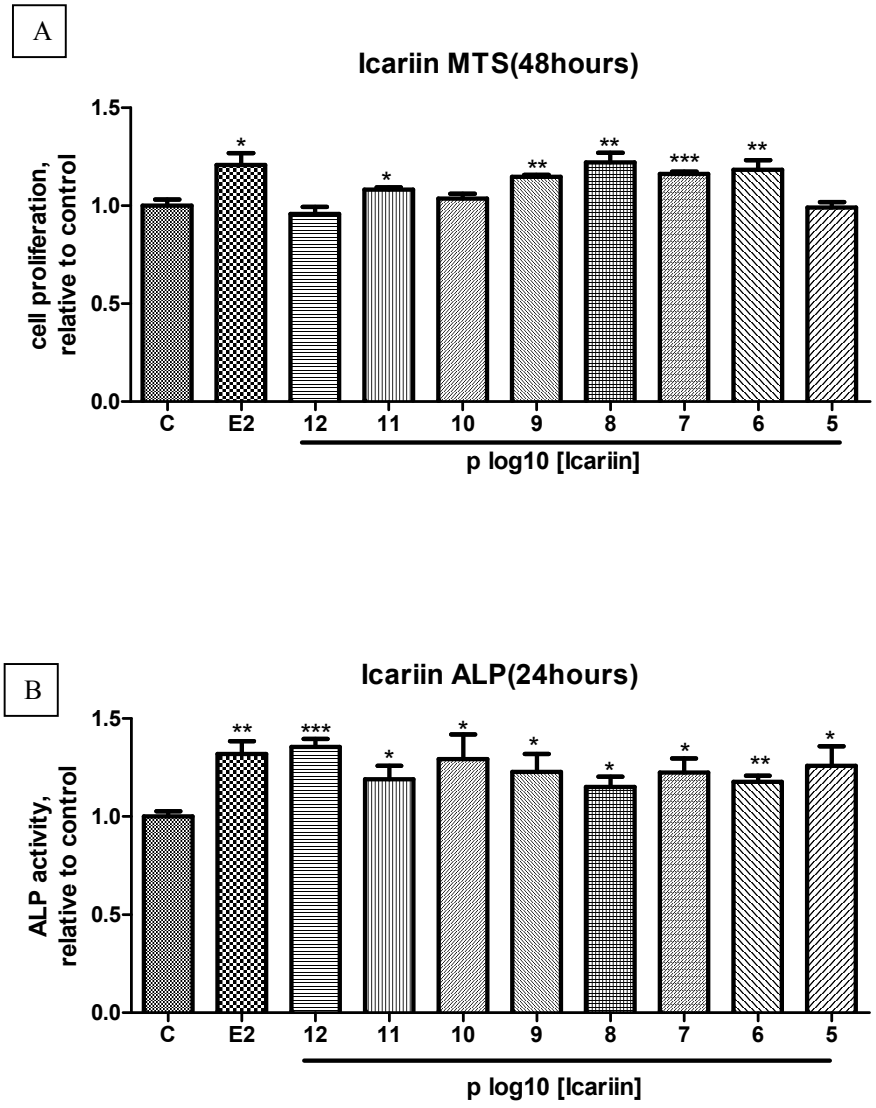


Figure 3.4: Effects of treatment with Icariin on UMR106 cell proliferation and ALP activity.

5,500 cells/ well were cultured in 96-well microtiter plate and were subjected to drug treatments for 24/48 hours. **(A)** Cell proliferation rate was assessed by MTS assay. **(B)** Cell differentiation was indicated by ALP activity in the assay normalized with total protein content (OD_{595}) in Bradford assay. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M) or Icariin (10^{-12}~10^{-6}M). Results were obtained from two experiments (n=6) and expressed as mean ± SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

Genistein

The effects of genistein on cell proliferation and differentiation varied vastly with the concentration applied in the treatment. At the lower end of the concentrations tested, i.e. 10^{-12}M to 10^{-8}M, genistein generally had a positive impact on the proliferation of UMR106 cells. A 1.32-fold increase ($P<0.01$ vs vehicle) in cell proliferation was recorded for genistein at 10^{-9}M in UMR 106 cells, and the effect was diminished to 1.18-fold increase ($P<0.05$) in response to 10^{-8}M treatment. For treatment at higher concentrations, 10^{-7}M and 10^{-6}M genistein failed to induce positive effects in UMR 106 cells and no detectable difference in cell proliferation were found when compared with the vehicle. Treatment of UMR cells with 10^{-5}M genistein resulted in a significant inhibition on cell growth, which was 0.72-fold ($P<0.001$ vs vehicle).

For ALP activity, the effects of genistein on differentiation of UMR106 cells were found to be generally weak at most of the concentrations tested. A marked increase in ALP activity for around 1.3-fold ($P<0.01$) was observed when treated with 10^{-11}M genistein. However, ALP activity of genistein-treated group at most of the concentration tested showed no statistical difference with the control group and only induced a weak increase in the ALP activity at the concentrations of 10^{-9}M and 10^{-8}M .

Thus, the results showed that genistein could stimulate or inhibit the osteoblastic cell proliferation and differentiation and their effects were found to be biphasic and highly dependent on the dosages for the treatment.

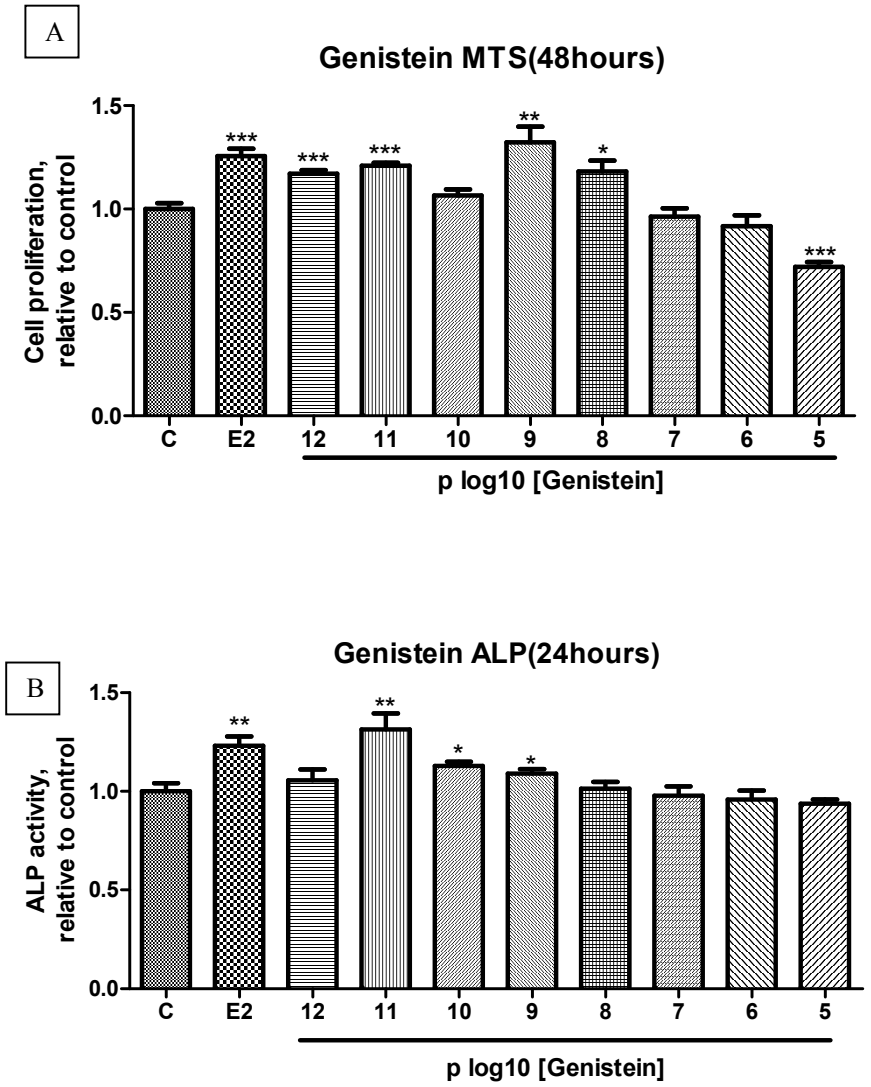


Figure 3.5: Effects of 24 hours and 48 hours treatment of Genistein on UMR106 cell proliferation and differentiation.

5,500 cells/ well were cultured in 96-well microtiter plate and were subjected to drug treatments for 24hours or 48 hours. **(A)** Cell proliferation rate was assessed by MTS assay. **(B)** Cell differentiation was indicated by ALP activity in the assay normalized with total protein content (OD_{595}) in Bradford assay. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M) or Genistein (10^{-9}~10^{-5}M). Results were obtained from two individual experiments (n=6) and expressed as mean $\pm$ SEM.* P<0.05;**P<0.01;***P<0.001 versus control.

3.3.2 Detection of GPR30 expression in UMR106 cells at mRNA level

Gel electrophoresis result

The mRNA of UMR106 cells was reverse-transcribed to cDNA and amplified by PCR using GPR30 specific primers. The size of the PCR products was found to be around 170bp, which was in close agreement with the reported product length (172bp) of the GPR30 primer pairs. The results suggested that UMR106 cells were likely to express GPR30 at least at the mRNA level.

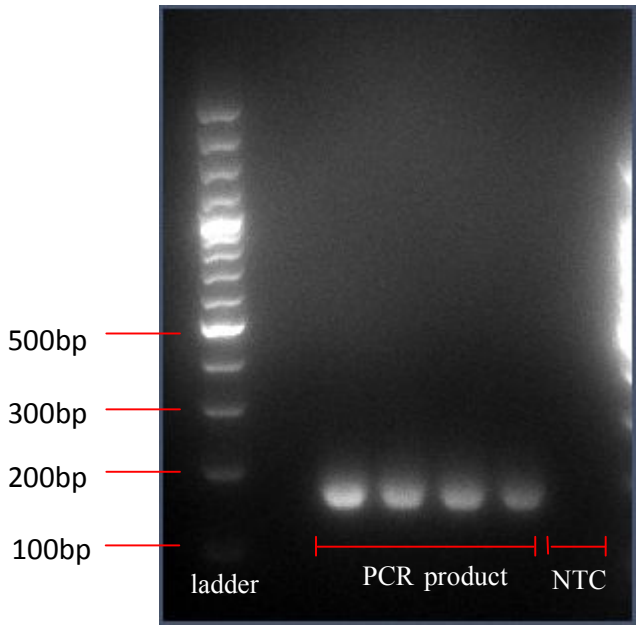


Figure 3.6: Gel Electrophoresis of PCR product

Ladder, PCR products and NTC (non-transcriptional control) were used to perform electrophoresis on 1.5% agarose gel.

3.3.3 Effects of ER and GPR30 antagonists on phytoestrogens-induced cell proliferation and differentiation of UMR106 cells

The optimum concentrations of the flavonoids which demonstrated the greatest potency in stimulating both UMR106 cells proliferation and differentiation were selected based on the results from the previous assays:

Table 3.5: Concentrations of the compounds used for antagonist experiment

Compounds	Concentration(M)	
	MTS	ALP
Naringin (NAR)	10^{-7}	10^{-7}
Icariin (ICA)	10^{-8}	10^{-12}
Genistein (GEN)	10^{-9}	10^{-11}
17β-Estradiol(E2) – positive control	10^{-8}	10^{-8}

The results demonstrated the effects of flavonoids in the presence or absence of ICI182780 (ER antagonist) or G15 (GPR30 antagonist) on the cell proliferation and differentiation of UMR106 cells.

Pre-treatment of ICI182780 and G15 with naringin, icariin and genistein

Figure 3.7 showed that the stimulatory effects of naringin, icariin and genistein at optimum dosages were antagonized by co-treatment with ICI and G15. After incubation for 24 or 48 hours, ICI as well as G15 effectively blocked the stimulatory effects on cell differentiation and proliferation of naringin, icariin and genistein-treated cells. Pre-treatment of cells with G15 prior to flavonoids treatment decreased cell proliferation rate back to the control level (around 0.92-fold for genistein) or even lower (around 0.86-fold for naringin and icariin). As for ALP activity, ICI effectively antagonized the stimulatory effects of estrogen as well as all 3 tested compounds in UMR 106 cells ($P<0.005$). Co-treatment with G15, on the other hand, were able to significantly reduce the effect of estrogen, icariin and genistein on cell differentiation ($P<0.05$) but demonstrated minor effect on naringin-induced cell differentiation in UMR 106 cells.

These findings suggested that the actions of naringin, icariin and genistein might involve both ER as well as via the interaction with the membrane-bound GPR30 receptor.

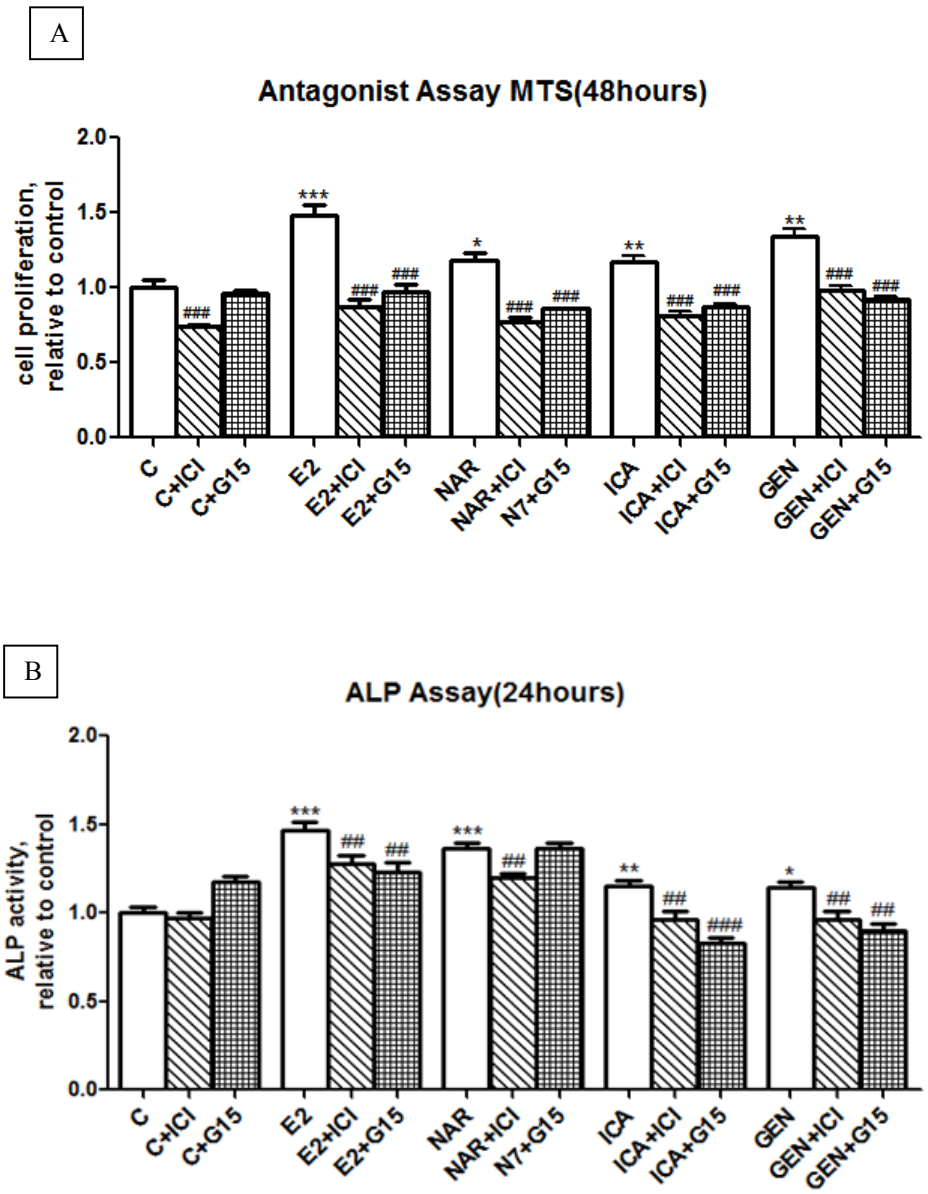


Figure 3.7: Effects of ICI182780 and G15 on stimulatory effects of flavonoids on UMR106 cell proliferation and differentiation.

UMR106 cells were subjected to drug treatments with or without blockers for 24/48 hours. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M) or Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-8}M) or Genisten (GEN, 10^{-9}M) in the presence or absence of ICI/G15. **(A)** Cell proliferation rate was assessed by MTS assay. **(B)** Cell differentiation was indicated by ALP activity in the assay normalized with total protein content (OD_{595}) in Bradford assay. Results were obtained from 2 individual experiments (n=6) and expressed as mean ± SEM.

*P<0.05;**P<0.01;***P<0.001 versus control.

#P<0.05 ; ##P<0.01###P<0.001 versus treatment group without antagonist addition.

3.3.4 Effects of flavonoids in the presence or absence of G15 on OPG/RANKL expression ratio in UMR106 cells

UMR106 cells were seeded in 6-well plate. After treatment with vehicle, 17β-estradiol (10^{-8}M), Naringin (10^{-7}M), Icariin (10^{-8}M) or Genisten(10^{-9}M) with or without G15 pre-incubation for 48 hours, total RNA was extracted and reverse transcribed into cDNA. Real time PCR was performed with the specific primer pairs for the particular gene to measure the mRNA expression of the gene.

The results showed that the mRNA expression of OPG was significantly up-regulated in UMR106 cells treated with E2 and all three flavonoid compounds. Treatment with E2, icariin and genistein showed no significant effect on the mRNA expression of RANKL, while naringin was able to up-regulate RANKL to 1.26-fold ($P<0.05$ vs control). There was a significant increase in OPG/RANKL ratio in UMR106 cells upon treatment with estrogen (1.32-fold increase, $P<0.001$), naringin (1.64-fold increase, $P<0.001$), icariin (1.52-fold increase, $P<0.01$) and genistein (1.94-fold increase, $P<0.001$) for 48 hours (vs control). These results suggested that naringin, icariin and genistein exerted suppressive effects on osteoclastogenesis by modulating OPG expression and OPG/RANKL ratio in osteoblasts.

Pre-treatment with G15 showed no antagonizing effects on the actions of E2 and all compounds tested in up-regulating the OPG expression and OPG/RANKL ratio in UMR 106 cells. Interestingly, treatment of cells with G15 alone led to a significant increase in the OPG/GAPDH expression (1.5-fold, $P<0.05$, vs control) and OPG/RANKL ratio (1.5-fold, $P<0.01$vs control). A minor increase in RANKL expression in UMR 106 cells was found in E2 and genistein-treated group with G15 pre-treatment.

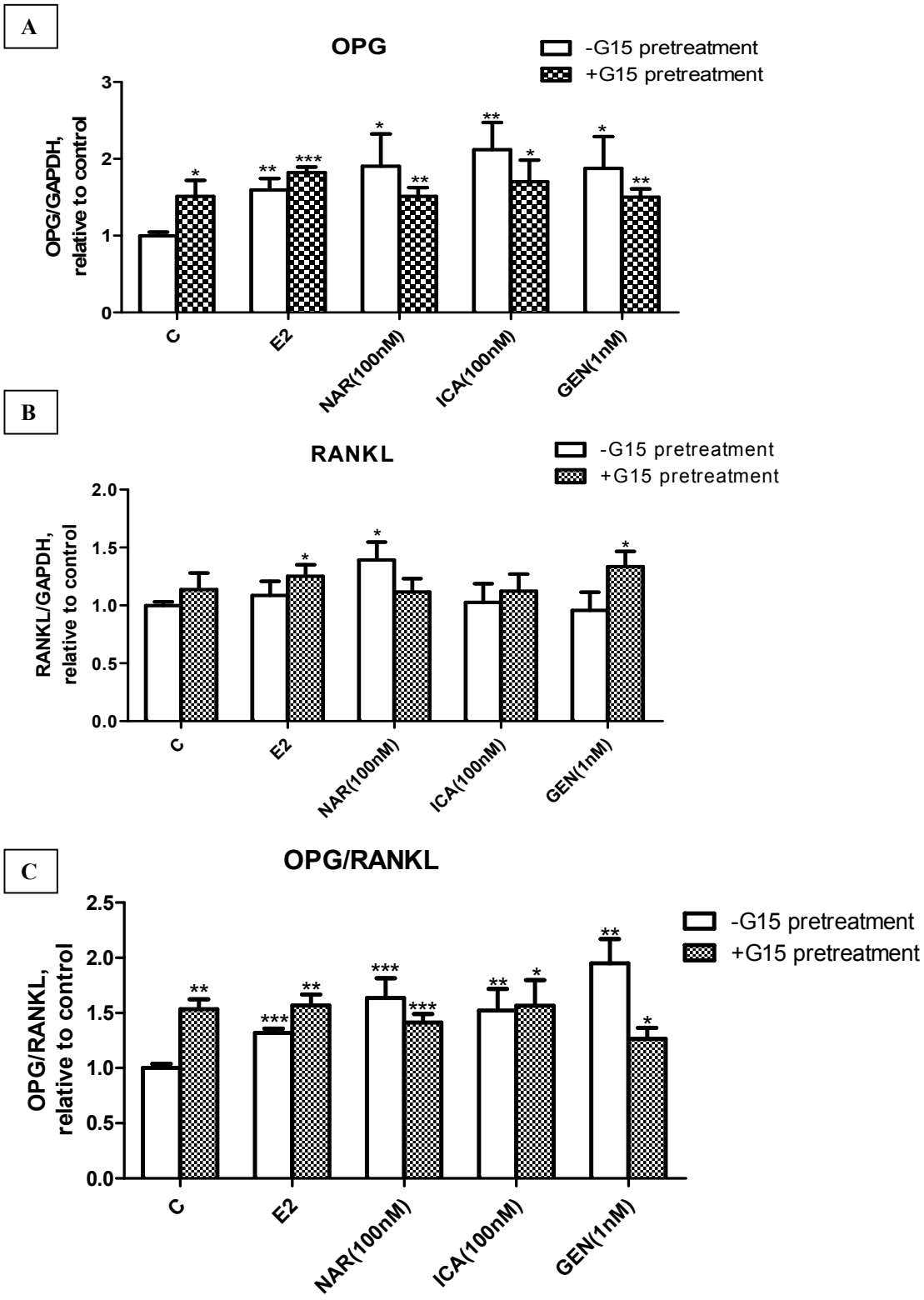


Figure 3.8: Effects of flavonoids treatment with or without G15 on expression of OPG, RANKL and OPG/RANKL ratio.

UMR106 cells were subjected to drug treatments in the presence or absence of G15 for 48 hours. For the antagonist group, G15 (10^{-6}M) were added into the wells and pre-incubated for 20 minutes before drug treatment. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M), Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-8}M) or Genisten (GEN, 10^{-9}M) for 48 hours. After treatment, Total RNA was isolated and reversed transcribed into cDNA. Real time PCR was performed to quantify the mRNA level of the respective genes. The mRNA expression of **(A)** OPG and **(B)** RANKL were normalized with the expression of the housekeeping gene GAPDH for each sample, and **(C)** OPG/RANKL ratio were calculated**.** Results were obtained from 2 individual experiments (n=4 or 5) and expressed as mean $\pm$ SEM.

* $P<0.05$;**$P<0.01$;***$P<0.001$ versus control.

3.4 Discussions

In the present study, the anabolic effects of naringin, icariin and genistein on rat osteoblastic UMR106 cells and their dependency to the estrogen receptors to exert these effects were systematically evaluated. It was important to note that this study was not aiming at comparing the potency among these three flavonoids but rather on investigating the bone protecting effects and the possible signaling routes exerted by each of them.

Previous studies have revealed that naringin exerts direct stimulatory effects on cell proliferation and differentiation in several osteoblast-like cell models (UMR106 cells of rat, MC3T3-E1 cells of mice) (Wong et al. 2006; Wu et al. 2008; Pang et al. 2010) and promotes osteogenic differentiation and proliferation in human bone mesenchymal stem cells (BMSC) (Dai et al. 2009). The result of the present study agreed with these findings and clearly demonstrated a potent effect of naringin on stimulating proliferation and ALP activity in UMR106 cells at a broad range of concentrations (10^{-10} to 10^{-6}M). Besides, it was found that the effect of naringin was following a dose-dependent trend which showed the most stimulatory result at concentration of 10^{-7}M in both the cell proliferation and differentiation of UMR106. The promoting effects of naringin could be extended to the concentrations as low as 10^{-10}M, indicating that naringin has a high potency in enhancing osteoblastic cells function. It is interesting to note that the anabolic effects of naringin attenuated with the increase in concentration after they peaked at 10^{-7}M, and its effects on both proliferation and ALP activity subsided at 10^{-5}M. Similar result was also reported by studies on bone marrow stromal cells (BMSCs) which demonstrated that a higher concentration of naringin treatment led to a stunted cell growth and even “seriously poisonous” to the BMSCs, leading to the decrease in the proliferation rate (Dai et al.

2009; Li et al. 2013). Thus, it was possible that the therapeutic effects of naringin on bone would be masked by the cytotoxic effects to the cells at a high concentration, though the mechanism underlying the effects remained unclear.

Research on Herba Epimedii extracts and its most potent active ingredient, icariin, demonstrated their enhancing effects on osteogenic functions in UMR106 cells (Xie et al. 2005; Mok et al. 2010). The osteoprotective potency of icariin had also been shown in various other osteoblastic cell lines such as primary human osteoblasts derived from mesenchymal stem cells and pre-osteoblastic MC3T3-E1 cells (Chen et al. 2007; Yin et al. 2007). The result of present study clearly demonstrated a promoting effect of icariin (10^{-12}M to 10^{-6}M) on proliferation and ALP activity in UMR106 cells at most of the concentrations. However, the concentrations of icariin that showed higher potency in stimulating cell proliferation would not necessarily be the most potent concentration for inducing UMR106 cells differentiation, and vice versa. A significant promoting effect of icariin on osteoblastic cells proliferation was shown at a higher concentration (10^{-9} to 10^{-6}M) but not at the concentrations in picomolar scale (10^{-12}, 10^{-10}M). On the contrary, the the lowest concentration of icariin tested in the experiment, i.e. 10^{-12}M was fount to be most potent for inducing ALP activity in UMR106 cells. The effect of icariin in promoting cell differentiation was found to be more potent than its effect in cell proliferation as all concentrations of icariin shown significant positive effect in ALP activity after treatment for 24 hours ($P<0.05$ vs control) . The findings were similar to our previous study by Mok et al. on UMR106 cells (Mok et al. 2010), and some other studies even found that icariin at certain concentration exhibited inhibiting effect on cell proliferation but significantly promoted differentiation of rat primary osteoblasts (Zhang

et al. 2008). The high potency of icariin in promoting osteogenic differentiation has been attributed to its action in stimulating bone morphogenetic proteins (BMP) signaling and Runx2 expression in osteoblastic cells (Zhao et al. 2008).

In contrast with the flavonol glycosides which exhibited relatively consistent positive effects on cell proliferation and differentiation among various osteoblastic cell types and in a broad range of concentrations, it was found that the effect of genistein was rather cell type and concentration-specific. In SaOS-2 cells, treatment with genistein at concentration of 10^{-8} M to 10^{-6}M did not alter the cell proliferation rate but did induce the ALP activity significantly in a dose-dependent manner (Chen et al. 2006). Treatment with genistein on human MG63 osteosarcoma osteoblasts, on the other hand, demonstrated a significant reduction on both cell proliferation and alkaline phosphatase activity in the range of concentration from 2.5uM ($2.5x10^{-6}$M) to 30uM (Morris et al. 2006).

The present study demonstrated the double-edge effect of genistein on UMR106 cells. At a lower concentration range (10^{-12}M to 10^{-8}M), genistein treatment generally demonstrated positive effects on cell proliferation; while at a higher concentration (10^{-5}M), genistein significantly inhibited the cell proliferation rate to about 0.86-fold (vs vehicle). Positive effects of genistein on osteoblastic differentiation in UMR 106 cells were also observed only at the lower concentration tested (10^{-11}M to 10^{-9}M), although the effects of genistein on ALP activity were generally weak in UMR106 cells. This biphasic effect of genistein on cell growth were also reported in breast cancer MCF7 cells (Wang et al. 1996). The author suggested that apart from being an estrogen-like isoflavone, genistein has another role as an inhibitor for receptor tyrosine kinases (RTK) (Akiyama et

al. 1987) and topoisomerase. Thus, the anti-proliferative effects of genistein at a higher concentration might be in part attributed to its role as a RTK inhibitor instead of a phytoestrogen, leading to the decrease in the growth rate of UMR106 cells.

Recent studies indicated that the effects of phytoestrogens in osteoblastic cells are not only exerted via ER-mediated genomic pathway but also via rapid, nongenomic cellular signaling pathway. However, the non-genomic estrogenic signaling as well as the rapid cellular signaling mediated by GPR30 on osteoblastic cells, has yet to be explored. Although the expression of GPR30 was reported in rat calvarial preosteoblasts (Teplyuk et al. 2008) and other osteosarcomas such as U2OS cells (Krum et al. 2008), the expression of GPR30 estrogen receptor in UMR106 cell line has not yet been reported. The present study verified the mRNA expression of GPR30 in the UMR106 cells.

The result of the antagonist assay demonstrated that the stimulatory effects on osteoblasts by naringin, icariin and genistein were also dependent on the GPR30-mediated signaling system in addition to the ER-dependent pathway. Co-treatment with ICI182780 (ER antagonist) as well as G15 (GPR30 antagonist) effectively blocked the stimulatory effects of all three phytoestrogens on cell proliferation and differentiation, suggesting the involvement of both receptors in the anabolic action of phytoestrogens on osteoblasts.

Another marker for anabolic effects of a particular compound in bone is its ability to up-regulate the ratio of OPG/RANKL. As described earlier, the increase of ratio between the expression levels of OPG and RANKL in osteoblasts indicates the ability of the particular phytoestrogen in modulating osteoclastogenesis. In the present study, we attempt to examine whether GPR30 is directly involved in the modulation of these 2

genes and also their ratio by estrogen and also our phytoestrogen compounds. Our results indicated that estrogen, naringin and icariin significantly up-regulated the mRNA expression of OPG and the ratio of OPG/RANKL. More importantly, it was found that the pre-incubation with G15 before treatment with phytoestrogens did not antagonize the stimulatory effect of the flavonoids as well as estradiol on OPG mRNA expression and OPG/RANKL ratio. In fact, treatment with G15 alone could significantly up-regulate the OPG/RANKL ratio. This was totally different from the effect of ER blocker ICI which effectively blocked the effects of estrogenic compounds in increasing OPG/RANKL ratio (Mok et al. 2010; Pang et al. 2010). One possibility is that there are different or even opposite role between classical ERs and GPR30 in modulating the gene expression of OPG and RANKL. Another possibility is that GPR30 is actually not involved in the OPG/RANKL ratio regulation, and the response of G15 treatment was in fact due to its effect acting on somewhere else. At this stage, therefore, it is hard to conclude that GPR30 has a role in modulating osteoclastogenesis or not.

Chapter 4

Characterization of estrogenic signalling pathways of phytoestrogens in UMR106 cells

4.1 Introduction

We have demonstrated that naringin, icariin and genistein were able to induce estrogen-like anabolic effects on osteoblastic cells, and their actions were shown to be dependent on the activation of estrogen receptors. In this chapter, we sought to investigate the underlying signaling mechanisms utilized by these compounds to exert their estrogenic effects, and to what extent do they resemble E2 or differ from it.

As introduced, the molecular mechanisms of estrogenic signaling could be exerted via four major pathways: ligand-dependent genomic pathway, ERE-independent genomic pathway, ligand-independent activation of ERs, and non-genomic rapid cellular signaling **(Figure 4.1)**. The most direct relationship between the compounds and nuclear ERs is the signaling via ligand-ER-ERE-mediated gene transcription. It was well characterized that some phytoestrogens including genistein served as a weak agonist for ERα and ERβ (Mueller et al. 2004). However, to our knowledge, few studies have been conducted to investigate the binding affinities of naringin and icariin to the estrogen receptors. In this study, the abilities of the flavonoids to displace radio-labeled E2 in binding recombinant human estrogen receptors ERα and ERβ were examined and measured quantitatively by competitive binding assays. The outcomes of binding to ER to the genomic signaling were validated with the abilities of these flavonoids to activate the estrogen-responsive transcription in osteoblasts. In this study, UMR106 cells were transiently transfected with ERE-luciferase reporter plasmid together with plasmids encoding ERα or ERβ to investigate the actions of flavonoids on ERE-mediated transcription via each ER subtype.

The ligand-independent phosphorylation of ERα could be initiated as rapid as 5 minutes at Serine 118, a site at AF-1 domain that can be triggered by various extracellular

signals without the presence of estrogen (Kato et al. 2000). The previous studies of our group have shown that naringin and icariin could activate ERα phosphorylation at Ser118 in UMR106 cells (Mok et al. 2010; Pang et al. 2010), but the actions of genistein in ERα phosphorylation in osteoblasts remained unknown. In this study, UMR106 cells were transiently transfected with plasmid encoding ERα and the abilities of each compound to induce the phosphorylation of ERα were validated.

It was now well accepted that the effects of estrogen in cells are not only constricted to its ability to promote ERE-dependent gene transcription on a long term basis, but also its ability to rapidly activate various secondary messenger signaling cascades, including the accumulation of cAMP, generation of inositol phosphate and its downstream kinases (PI3K, Akt) and activation of mitogen-activated protein (MAP) kinases, Erk-1 and Erk-2 (Filardo et al. 2000). These nongenomic estrogenic signaling pathways were found to be associated with the recruitment and activation of cell-surface signaling proteins to generate rapid tissue responses. In breast cancer cells, for instance, the endogenous membrane ERs cross-talk with the EGF, ErbB2, and IGF-I receptors, result in downstream signaling of ERK/MAPK and PI3K/Akt kinases. ERK and PI3K, in turn, phosphorylate discrete residues of the endogenous ERs to activate them, which subsequently up-regulating the transcriptional activity or stability (Levin 2005).

As both the ligand-independent activation of ERα and the nongenomic signaling of E2 were linked with the MAPK and PI3K/Akt rapid cellular signaling systems, we evaluated the effects of the flavonoids in regulating the rapid activation of the key members in these signaling cascades in UMR106 cells. For MAPK signaling, we examined the actions of phytoestrogens on rapid phosphorylation of MEK1/2 and

ERK1/2, while for PI3K/Akt signaling, the actions of the compounds on Akt phosphorylation as well as protein expression of PI3K and Akt within an hour time course were studied.

It was suggested that nongenomic activation of estrogen receptors by 17β-estradiol also involves the recruitment and activation of heterotrimeric G proteins. The Gαs subunit stimulates the activity of adenylyl cyclase, leading to rapid production and accumulation of intracellular cAMP, a secondary messenger widely utilized in cell signaling. The increase in cAMP serve as a signal to initiate PKA phosphorylation of the CRE-binding (CREB) protein, which in turn bind to cAMP response element (CRE) to initiate the CRE-dependent gene transcription (Marinissen et al. 2001). In this study, we investigated the rapid effects of E2 as well as the flavonoids administration on intracellular cAMP accumulation in UMR106 cells, and the results were validated with the ability to induce CRE-luciferase activity of each compound in the transfected cells.

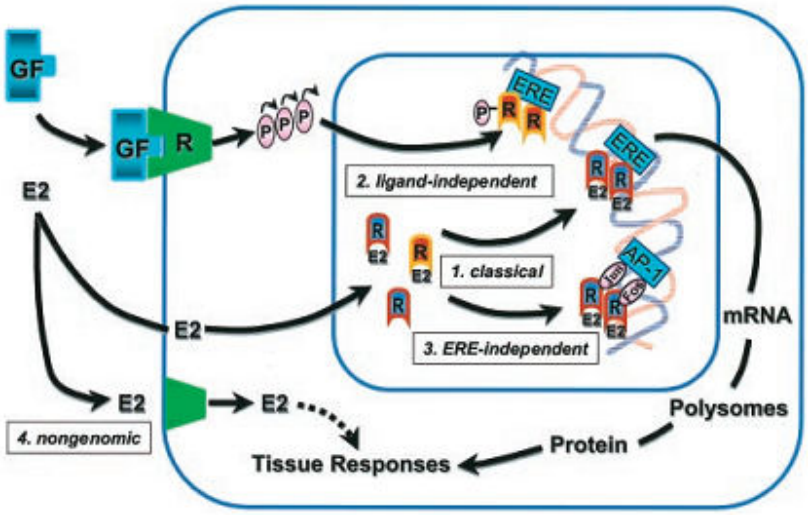


Figure 4.1: The possible mechanisms of estrogenic signaling (Hall et al. 2001)

The signaling by estrogenic compounds via estrogen receptors were exerted through the 4 main pathways: 1. Classical genomic pathway that required ligand binding to ERs and nuclear translocation of activated ER dimers to bind EREs in promoter region of the estrogen-responsive genes, thereby regulating their transcription. 2. Ligand-independent activation of estrogen receptors via a series of kinases signaling cascades induced by extracellular signals such as growth factors. 3. ERE-independent genomic pathways that include the actions of ligand-activated ERs in regulating alternative transcription targets containing response elements such as AP1 or Sp1. 4. Nongenomic estrogen signaling associated with activation of cell-surface receptors to induce rapid cellular signaling such as MAPK, PI3K/Akt signaling or cAMP-mediated signaling systems.

4.2 Methodology

4.2.1 Competitive binding assay with purified ERα and ERβ

96 well filter microtiter plates (Millipore, USA) was used in the assay. Each compound as well as 17β-estradiol was diluted with 1:1 DMSO : Assay buffer (50mM Tris, 10% glycerol, pH 8.0, 0.3mg/ml ovalbumin,0.01M mercaptoethanol) to seven concentrations from 7×10^{-5} M to 7×10^{-11} M. 10 µl compound dilutions, 10 µl [^{3}H]E_2 (7×10^{-8} M) and 50 µlfull length recombinant human ERα or ERβ protein (1.0×10^{-9} M) (PanVera/Invitrogen Corp, Carlsbad, USA) were loaded into each well. The plate was mixed for 5 min and then incubated overnight (18-24 h) on ice. Hydroxyapatite (HAP) slurry (Bio-Rad Pacific Ltd, Hong Kong) was then added to each well, mixed and incubated 15 min at 0^{o}C. After that the incubation solution was pulled through the HAP and the membrane using 100% of the vacuum. The plate was washed twice with ice cold HAP wash buffer with the aid of vacuum. After the washes, HAP wash buffer was added into each well to resuspend the slurry and the solutions were transferred to a scintillation counting vial containing scintillation fluid (Fisher Scientific, USA). The radioactivity of each sample was expressed as disintegration per minute (dpm) measured by a liquid scintillation counter (Beckman LS6500 Scintillation Counter). The binding of [^{3}H]E_2 to ER in the presence of competitor was determined by subtracting the dpm from the nonspecific binding and expressed as percentage of total binding without competitor. The value of IC50, i.e. the concentration of compound with 50% of maximal competition was calculated by Graph Pad Prism Software. The relative binding affinity (RBA) to E2 was calculated as:

RBA= IC50 of E2/ IC50 of the competitor x 100.

Hence, the RBA of E2 would be 100% by definition.

4.2.2 Transient transfection and ER -mediated ERE-dependent luciferase activity assay

To investigate if the flavonoid compounds are able to induce Estrogen Response Element (ERE) transcriptional activities through the classical estrogen receptors, pERE-luc and plasmid encoding ER-α or ER-β were transiently transfected into UMR106 cells. pERE-luc (kindly provided by Dr. Vincent Giguere, McGill University) is a recombinant plasmid which contains ERE gene and a reporter gene which encodes firefly luciferase.

UMR106 cells were seeded and cultured in 24-well plate for 48 hours before replacing the growth medium with phenol-red free DMEM supplemented with 1% dextran-charcoal-stripped-serum (sFBS) without antibiotics. After 24 hours, the cells were co-transfected with 0.4ug ER-α or ER-β plasmid, 0.4ug pERE-luc **(Figure 4.2)**, and 0.01ug pRL-TK, an internal control Renilla Luciferase vector for normalization. The transfection into the cells was done by complexing the plasmids with Lipofectamine^{TM} 2000 reagent (Invitrogen) and incubated in phenol red-free DMEM without any supplement for 6 hours. Vehicle, estradiol (10^{-8}M), or the flavonoid compounds (10^{-10}M to 10^{-6}M) was then added followed by 24 hours incubation in 37°C. Then, the cells were lysed using Passive Lysis Buffer and Dual Luciferase Reporter Assay was carried out to perceive the activities of both the luciferases following the protocol (Promega, #TM040). The luminescent signals were detected using Glomax-20/20 Luminometer (Promega, Madison, USA). The ratio of first reading (firefly luciferase activity) and second reading (Renilla luciferase activity) reflected the activities of the receptors in activating ERE transcription level.

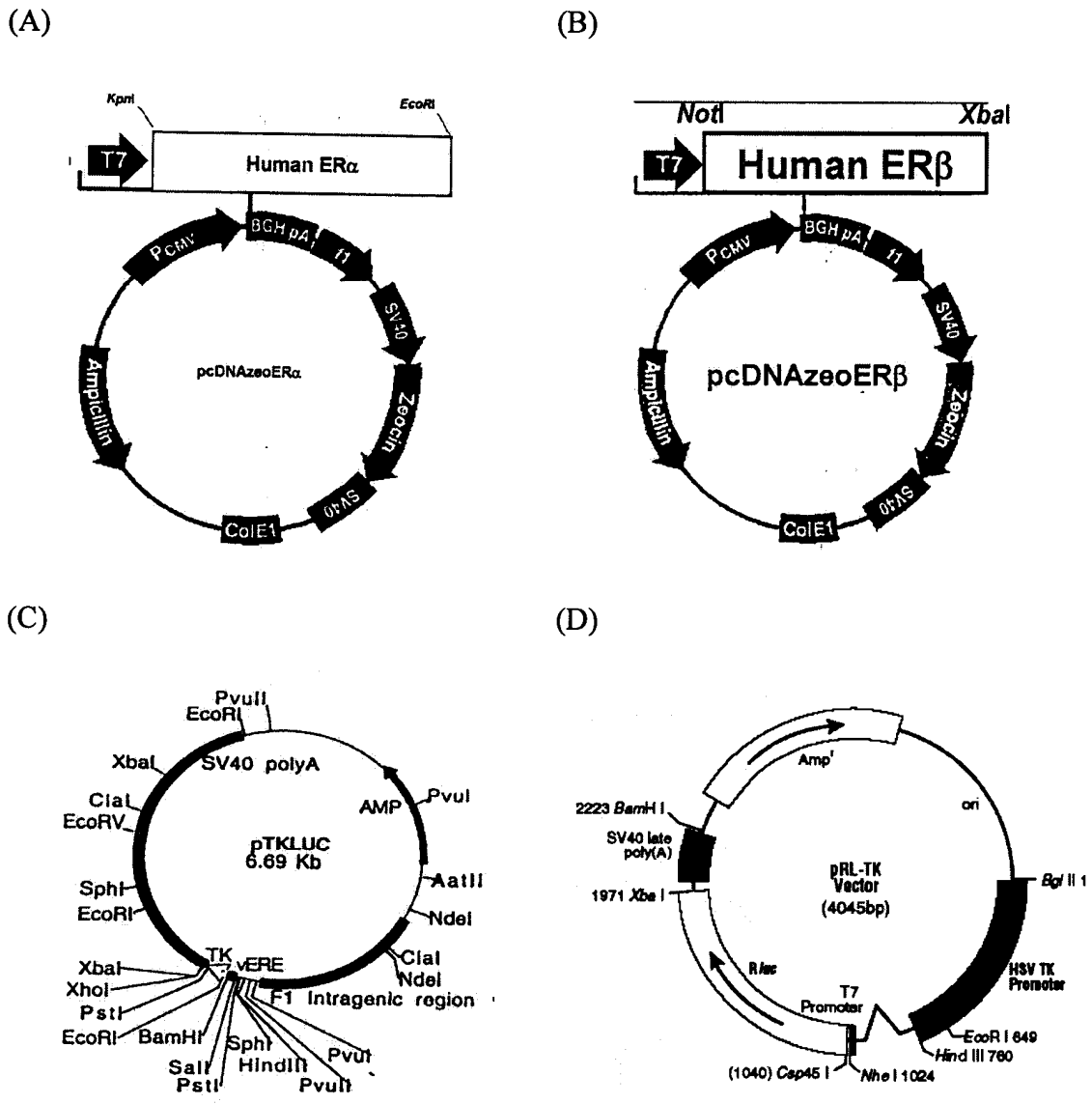


Figure 4.2: The vector maps of (A) ERα plasmid (B) ERβ plasmid (C) ERE promoter-containing luciferase plasmid and (D) pRL-TK vector.

4.2.3 Immunoblotting

The cells were seeded in 6-well plate with appropriate cell density as mentioned. After treatment, the cells were lysed in Nonidet P40 lysis buffer (20mM Tris-HCl, pH7.5, 150mM NaCl, 1mM CaCl2, 1mM MgCl2, 10% glycerol, 1% Nonidet P-40) supplemented with protease inhibitors (2ug/ml aprotinin, 2ug/ml leupeptin, 1mM PMSF, 1mM sodium orthovanadate, 10mM NaF). Protein concentrations were determined using Bradford method (Bio-Rad Laboratory, USA). Equal amount of protein lysates (50ug) were loaded and separated by running SDS-PAGE with 180V on 10% reducing gelfor approximately 1 hour. The gel was then transblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilin-P, Millipore Corp., MA, USA).

After blocking the non-specific binding sites with 10% skimmed milk for 1-2 hours, the PVDF blots were probed overnight at 4°C with the following primary antibodies: rabbit anti-human phospho-ERα at Ser118 (1:2000, Upstate), rabbit anti-human ERα(1:1000, Santa Cruz), rabbit anti-p85 PI3K (1:1000, Cell Signaling), rabbit anti-phsopho-Akt at Ser473 (1:1000, Cell Signaling), rabbit anti-Akt1/2/3 (1:1000, Santa Cruz), mouse anti-phospo-ERK (1:1000, Santa Cruz), rabbit anti-ERK1/2 (1:1000, Cell Signaling), rabbit anti-phospho-MEK1/2 at Ser218/Ser222 (1:1000, Santa Cruz), rabbit anti-MEK1/2 (1:1000, Santa Cruz)or mouse anti-β actin (1:2000, Abcam). The blots were then washed 3 times with TTBS followed by incubation with anti-rabbit (1:2000, Santa Cruz) or anti-mouse (1:2000, Cell Signaling Technology) IgG-HRP-conjugated secondary antibodies for 1-2 hours. ECL western blotting detection reagents (Clarity™ Western ECL Substrate, Bio-rad, USA) were added to the blots and the chemiluminescence of the bound antibodies were visualized and quantified by the Lumi-Imager F1 Workstation (Roche,

Manheim, Germany). The intensity of the signal in the band was expressed as Biochemical Light Units (BLU).

4.2.4 Intracellular cAMP measurement

UMR106 cells were seeded in 6-well plate and incubated for 48 hours before changing to steroid-starved medium. After 24 hours incubation in phenol red free DMEM supplemented with sFBS, vehicle, PTH(10^{-8}M), estradiol(10^{-8}M) or the optimal concentrations of compounds determined from MTS and ALP assays were applied into the wells. After the drug stimulation for 10 minutes, the cells were then lysed with 0.1M HCl and incubated at room temperature for 20 minutes. Using plastic cell scrapper, the cells were scrapped from the plate and transferred to a clean 1.5ml tube. The tubes were centrifuged at 1000g for 10 minutes and the supernatant were collected. cAMP concentration was directly measured by using Cyclic AMP EIA Kit(Cayman, USA) following the manual instructions. The absorbance of cAMP is read at 405-420nm by microplate reader (Bio-rad, USA) and the reading was normalized with protein concentration of each sample determined by Bradford protein assay. The cAMP concentration was calculated as pmol/mg protein.

4.2.5 CRE-dependent luciferase activity assay

pCRE-luc (kindly provided by Prof. Karl Tsim, The Hong Kong University of Science and Technology) which contains CRE gene and firefly luciferase reporter was transfected into UMR106 cells to investigate if the flavonoid compounds are able to induce cAMP Response Element (CRE) transcriptional activities via cAMP-dependent signaling. The transfection was performed as described above in ERE-dependent luciferase activity assay with minor modifications. Briefly, UMR106 cells were co-transfected with 0.25ug pCRE-luc reporter plasmid and 0.01ug pRL-TK plasmid. 6 hours following transfection, UMR106 cells were stimulated with PTH (10^{-8}M), estradiol (10^{-8}M) or the flavonoids (10^{-10}M to 10^{-6}M) for 10 minutes. The compound-containing medium was then replaced with phenol red free DMEM supplemented with 1% sFBS and P/S for further 24 hours incubation. The cells were then lysed with PLB and luciferase assays were performed as described.

4.2.6 Statistical Analysis

Results were expressed as mean $\pm$standard error of mean (SEM) and were analyzed by two-tailed unpaired Student's t-test between the drug-treated group and vehicle-treated control group using Microsoft Office Excel 2007.A value of $p < 0.05$ was considered significant. All graphs in the study were plotted using GraphPad Prism Version 4.00.

4.3 Results

4.3.1 Relative binding affinities of Naringin, Icariin and Genistein to ERα and ERβ

The study investigated the abilities of naringin, icariin and genistein to compete with [^{3}H]-E2 for binding to purified recombinant human estrogen receptors ERα and ERβ. The results showed that non-radiolabeled E2 displaced [^{3}H]-E2 with the IC50 values of 1.26×10^{-8}M for ERα and 1.39×10^{-8}M for ERβ. Both numbers were close to the theoretical value of 1×10^{-8}M, confirming the reliability of the assay.

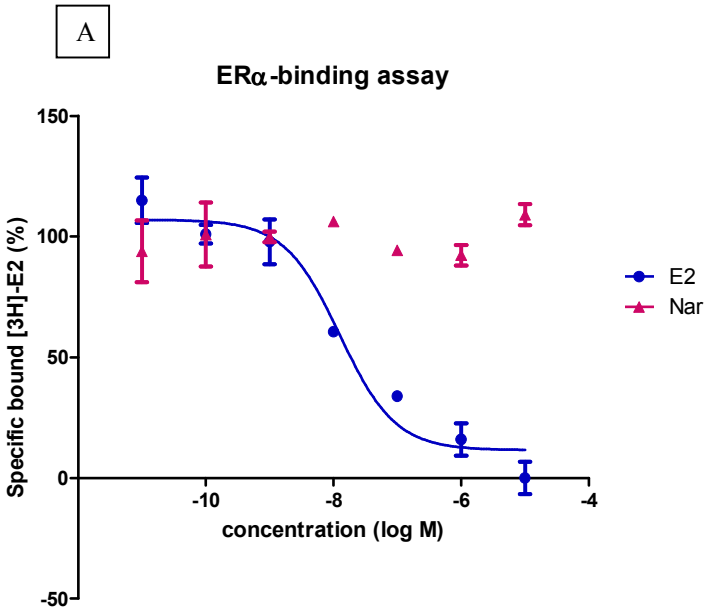
For naringin, there was no trend of displacement observed within the dosage of 10^{-11}M to 10^{-5}M for both ERα and ERβ. Less that 10% of displacement was observed for the range of concentrations, even at the highest concentration (10^{-5}M) which was a 1000-fold larger than the [^{3}H]-E2 (10^{-8}M).

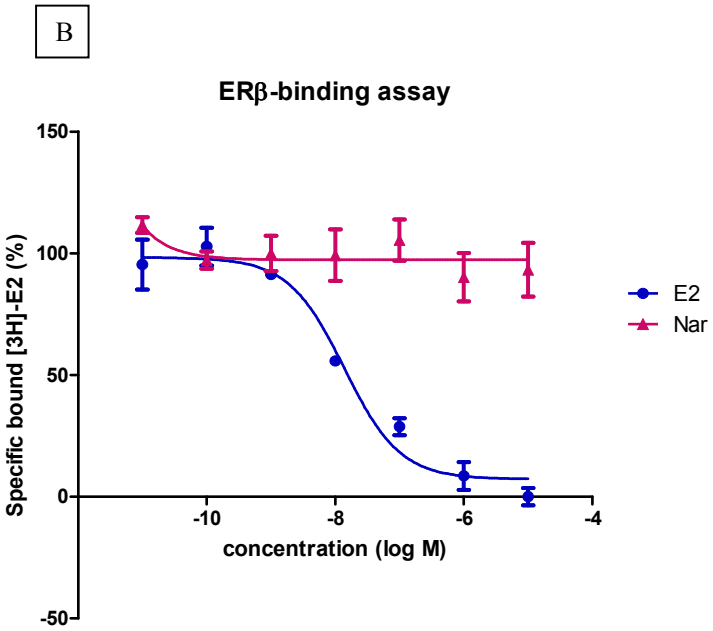
For icariin, no significant displacement was detected for concentrations up to 10^{-5}M. For affinity to ERα, all concentrations demonstrated less than 10% of displacement. Although some fluctuation were observed in the ERβ-binding assay, the displacement by icariin remained under 30% and did not demonstrated increasing trend with higher concentration.

Thus, the calculation of relative binding affinity (RBA) of naringin and icariin were not applicable as no apparent binding with ERs was observed for both compounds.

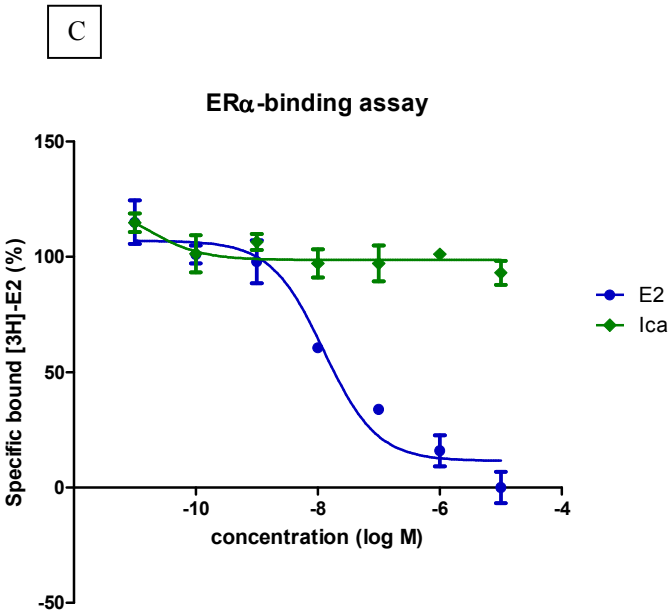
Our results showed that genistein displaced [^{3}H]-E2 for binding to both ERα and ERβ. Genistein bound to ERβ with greater affinity (IC50 = 2.39×10^{-8}M) than to ERβ (IC50 = 1.27×10^{-6}M). The RBA to ERα was 0.09% relative to E2, while the RBA to ERβ was significantly higher, i.e. 58.13% relative to E2.

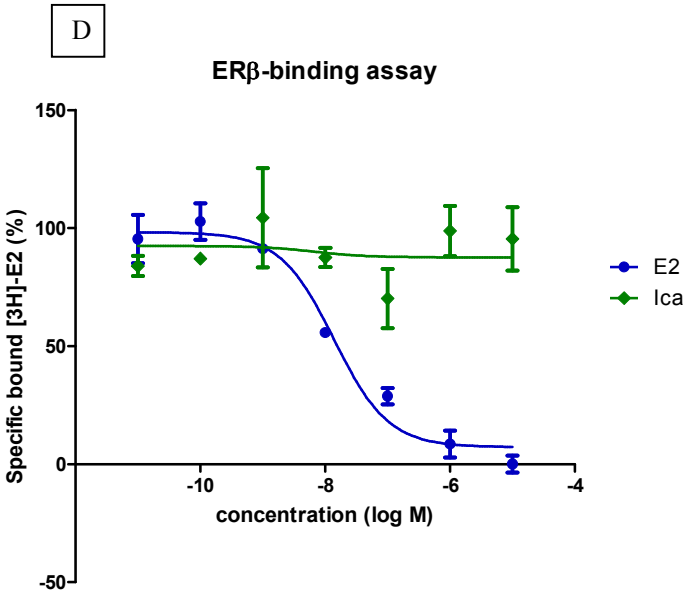
Naringin





Icariin





Genistein

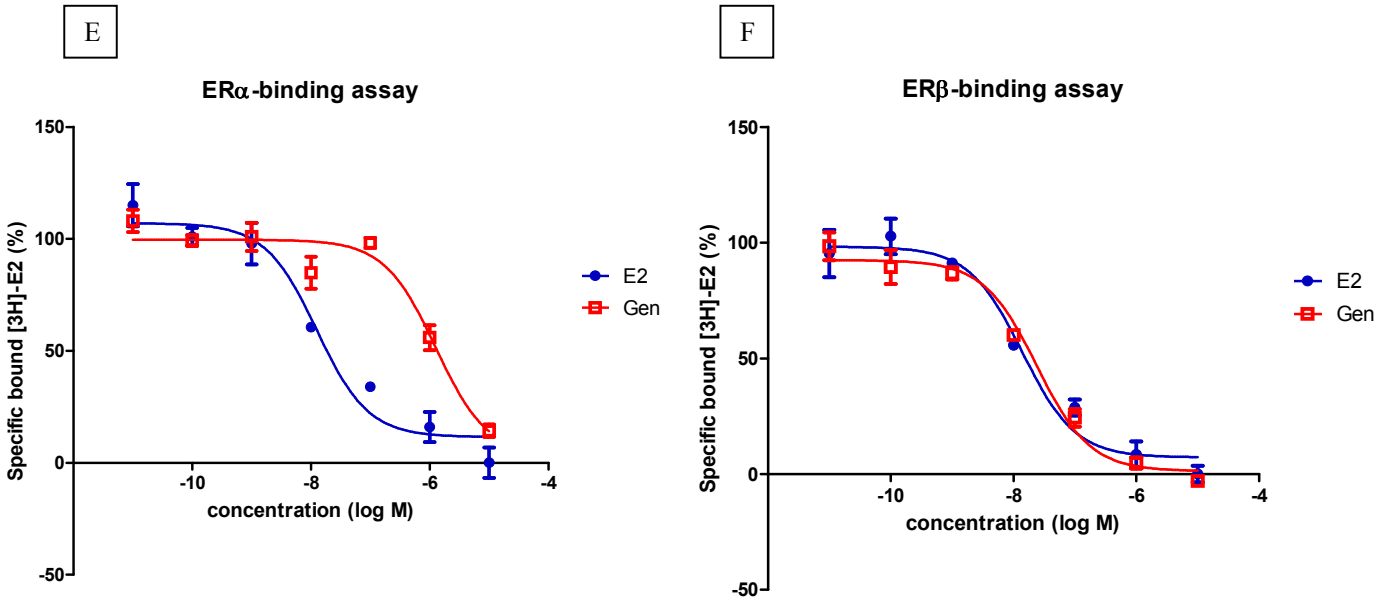


Figure 4.3: Competitive ligand binding assays of Naringin (A, B), Icariin (C, D) and Genistein (E, F) for binding to ERα and ERβ. Increasing concentrations of the competitors (10^{-11}M to 10^{-5}M) were added to measure the displacement of specific [^{3}H]-17β-estradiol binding to recombinant human ERα and ERβ proteins. Data was expressed as percentage specific bound [^{3}H]-E2 and obtained from at least two separate experiments (n=4).

4.3.2 Actions of flavonoids via genomic ER pathway

Effects of flavonoids on ERα or ERβ–mediated ERE-dependent luciferase Activity in UMR106 cells

In this study, effects of naringin, icariin and genistein on inducing transcriptional activities of ERE promoter-containing luciferase were investigated. Naringin at all concentrations tested (10^{-10}M to 10^{-6}M) were unable to stimulate ERE-dependent luciferase activity in UMR106 cells cotransfected with either ERα or ERβ. Treatment with icariin at most of the concentrations failed to induce ERE transcriptional activitiy via ERα and ERβ except at the highest concentration tested, 10^{-6}M, which led to mild elevation of the luciferase activity to 1.4-fold versus control ($P<0.05$) in ERα-mediated ERE-transcription.

In contrast, treatment with genistein significantly induced ERE-dependent luciferase activity in UMR106 cells via both ERα and ERβ, though preferentially via ERβ. Stimulation of ERα-mediated ERE-dependent luciferase activity in UMR106 cells required a higher concentration of genistein (10^{-7}M to 10^{-6}M) while a lower genistein dosage (10^{-9}M) was sufficient to significantly increase the ERβ-mediated ERE-dependent luciferase activity in compare to control (2.18-fold increase, $P<0.05$).

Together, the results indicated that naringin at all tested concentrations did not exert its anabolic effect on UMR106 cells via activation of ERE-dependent transcriptional events, while icariin did not induce ERβ-mediated ERE-transcription and only weakly induced ERα-mediated ERE-transcription at high concentration. The effects of genistein on UMR106 cells, on the other hand, involved the activation of ERE-dependent transcription via both ERα and ERβ with a higher potency for ERβ.

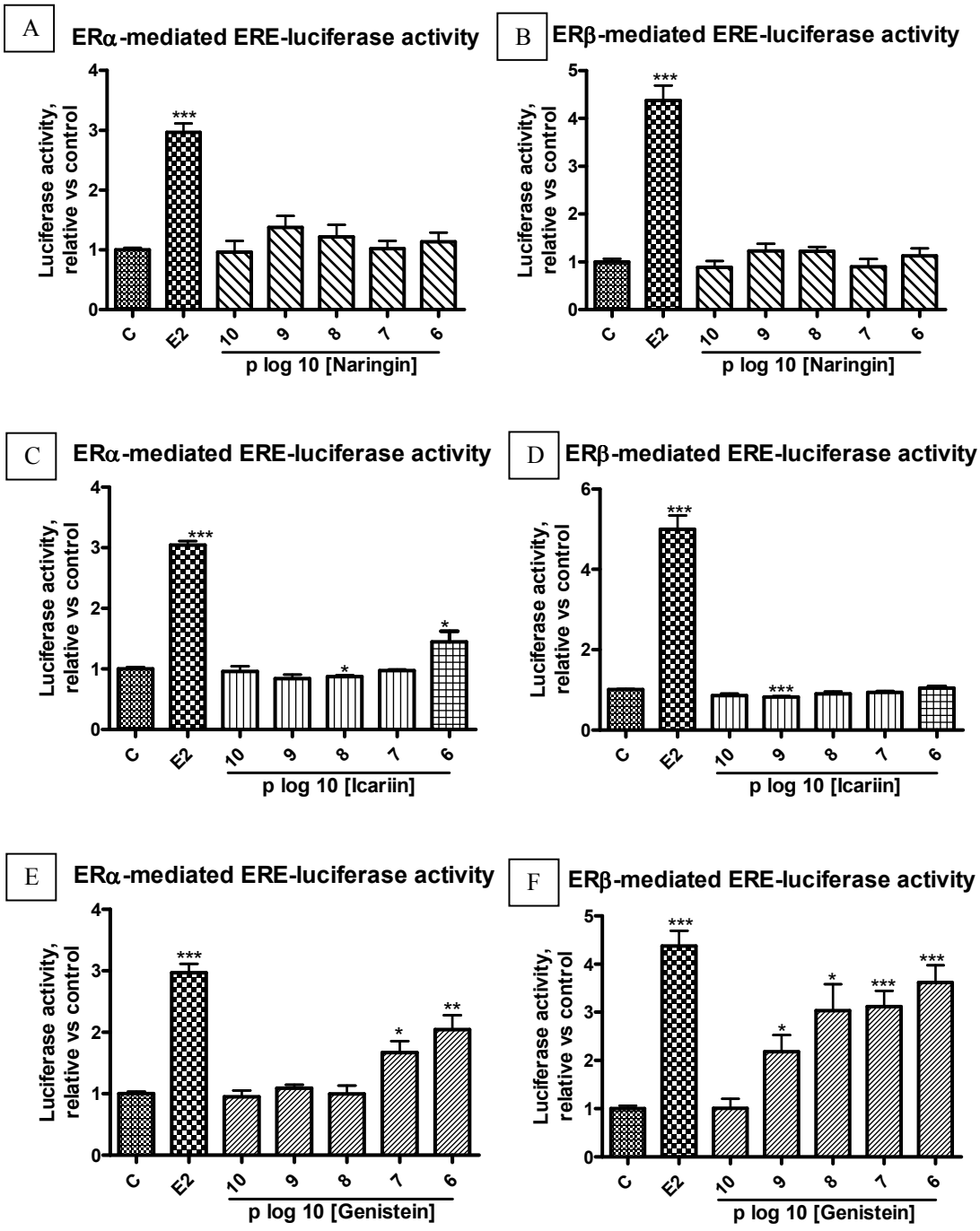


Figure 4.4: Effects of treatment with Naringin (A, B), Icariin (C, D) and Genistein (E, F) on inducing ERE-dependent luciferase activity in UMR106 cells.

UMR106 cells were co-transfected with 0.4ug ER-α or ER-β plasmid, 0.4ug pERE-luc, and 0.01ug pRL-TK with Lipofectamine 2000 and incubated for 6 hours. The cells were subjected to treatments with vehicle(C), 17β-estradiol (E2, 10^{-8}M), Naringin (10^{-10}M to 10^{-6}M),or Genisten(10^{-10}M to 10^{-6}M) for 24 hours. Firefly and Renilla Luciferase activities were measured sequentially with Dual Luciferase Assay reagents. Results were obtained from 2 individual experiments (n=4) and expressed as mean $\pm$ SEM. * P<0.05;**P<0.01;***P<0.001 versus control.

4.3.3 Effects of flavonoids on phosphorylation of ERα at Ser118 in UMR106 cells

The above results demonstrated that the actions of naringin and icariin was independent of direct binding to ERα and ERβ, and also failed to induce ERE-dependent transcription via both ERs in UMR106 cells. To determine if naringin, icariin and genistein could activate ERα indirectly with a mechanism independent of ligand binding, the degree of phosphorylation of ERα at serine 118 residues relative to total ERα was measured in transfected UMR106 cells treated with the compounds for 24 hours. As shown in figure 4.5, E2 and all three compounds significantly increased the ratio of pERα/ERα. Estradiol treatment led to 2.3-fold increase ($P<0.001$ vs control) in phosphorylation, while treatment with naringin, icariin and genistein significantly increased ERα phosphorylation by 1.45-fold ($P<0.001$), 2.48-fold ($P<0.01$) and 2.46-fold ($P<0.05$) versus control respectively. Thus, the result suggested that the flavonoids could induce activation of ERα via phosphorylation of serine 118 residue.

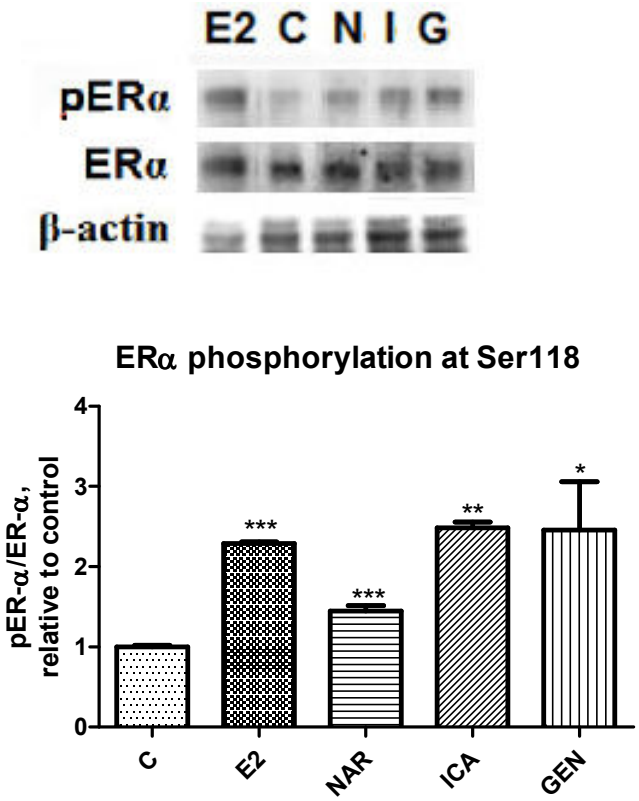


Figure 4.5: Effects of Naringin, Icariin and Genistein on phosphorylation of ERα at Ser118 in UMR106 cells.

UMR106 cells were co-transfected with 0.4ug ER-α with Lipofectamine 2000 and incubated for 6 hours. The cells were then subjected to treatments with vehicle(C), 17β-estradiol (E2, 10^{-8}M), Naringin(N, 10^{-7}M), Icariin (I, 10^{-7}M)or Genistein(G, 10^{-9}M) for 24 hours and the cell lysates were collected. After SDS-PAGE, the blots were probed with anti-phospho-ERα at Ser118 (pERα), anti-ERα and anti-β-actin primary antibodies.The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from three individual experiments (n=3) and expressed as mean $\pm$ SEM. * $P<0.05$;** $P<0.01$;*** $P<0.001$ versus control.

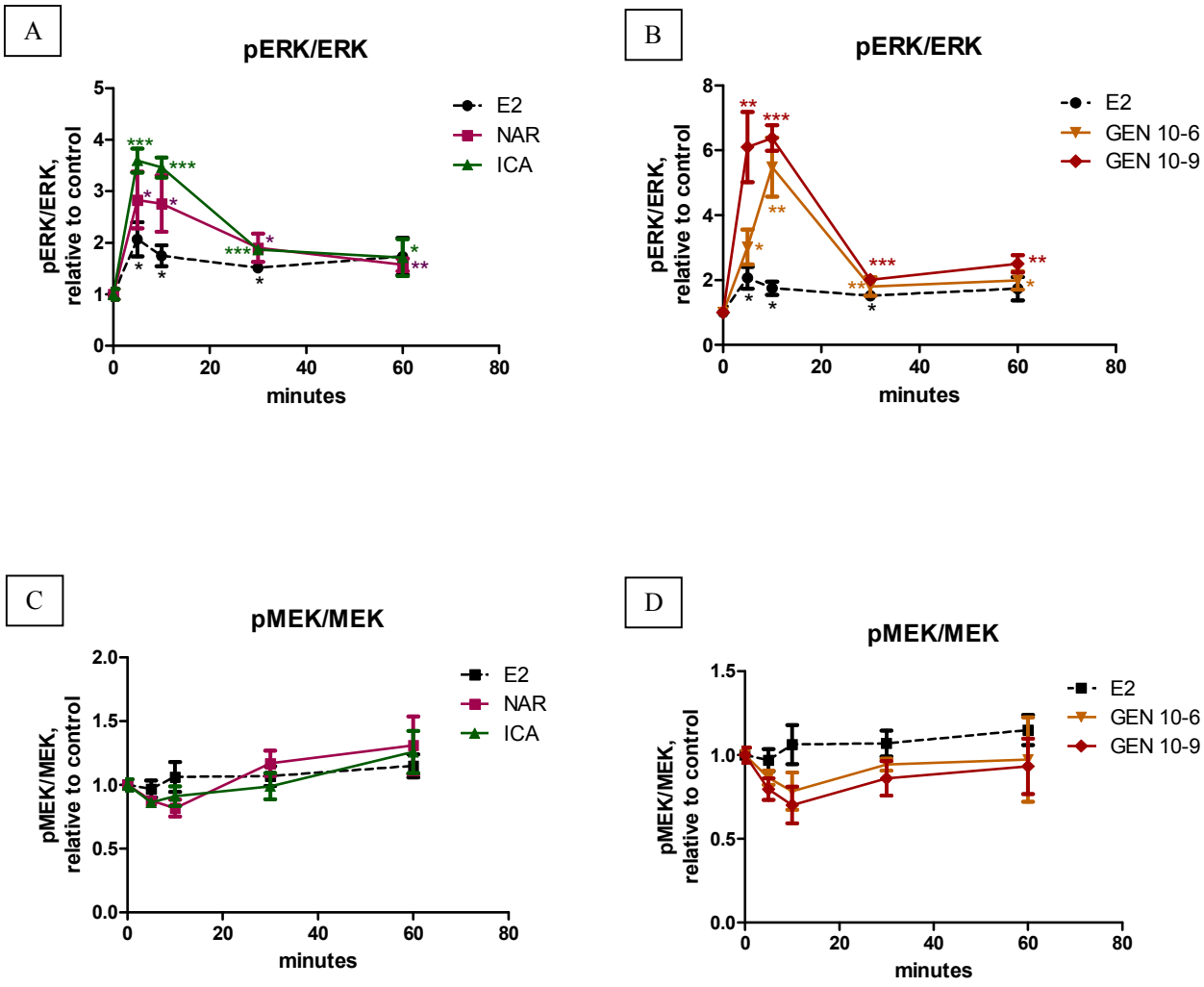
4.3.4 Effects of flavonoids on phosphorylation of ERK-1/2 and MEK of MAPK Pathway in UMR106 cells

As the phosphorylation of ERα at Ser118 was associated with a ligand-independent mechanism involving the MAPK pathway, we assess the abilities of the flavonoids to induce rapid activation of MAPK signaling cascades. The cells were treated with E2 (10^{-8}M), naringin (10^{-7}M), icariin (10^{-7}M) and genistein (10^{-6}M, 10^{-9}M)for different periods of time (5mins, 10mins, 30mins, 60mins) and their abilities to induce the phosphorylation of ERK1/2 and MEK1/2 were assessed by immunoblotting and normalized with the total expression of the respective proteins.

The results showed that treatment with E2 and all three flavonoids induced ERK1/2 phosphorylation in UMR106 cells as rapid as 5 minutes and sustained until at least an hour. Treatment with E2 led to stable and sustained stimulation of ERK1/2 phosphorylation at around 2-fold ($P<0.05$ vs control) throughout the time course from 5 minutes to 1 hour. On the other hand, the phosphorylation of ERK1/2 in response to flavonoids treatment was found to be dramatically ascended to reach their maximal at 5 to 10 minutes, in which they showed a higher degree of phosphorylation than E2 itself. Treatment with naringin and icariin for 10 minutes led to a 2.76-fold ($P<0.001$) and 3.46-fold ($P<0.001$) increase in pERK/ERK ratio, respectively. Treatment with 10^{-9}M and 10^{-6}M genistein for 10 minutes even led to a greater phosphorylation of ERK, which increased to 6.37-fold ($P<0.001$) and 5.5-fold ($P<0.01$), respectively. This was followed by a sustained level of phosphorylation at a degree similar to E2, i.e. 2-fold increase in pERK/ERK ratio for naringin, icariin as well as genistein at 30 minutes and 1 hour incubation. In contrast, treatment with E2, naringin, icariin and genistein did not alter the

phosphorylation level of MEK1/2 within 60 minutes.

The results showed that the estrogenic signaling of naringin, icariin and genistein in UMR106 cells involved the activation of MAPK signaling pathway via rapid and sustained phosphorylation of ERK1/2 without changing the degree of phosphorylation of MEK1/2.



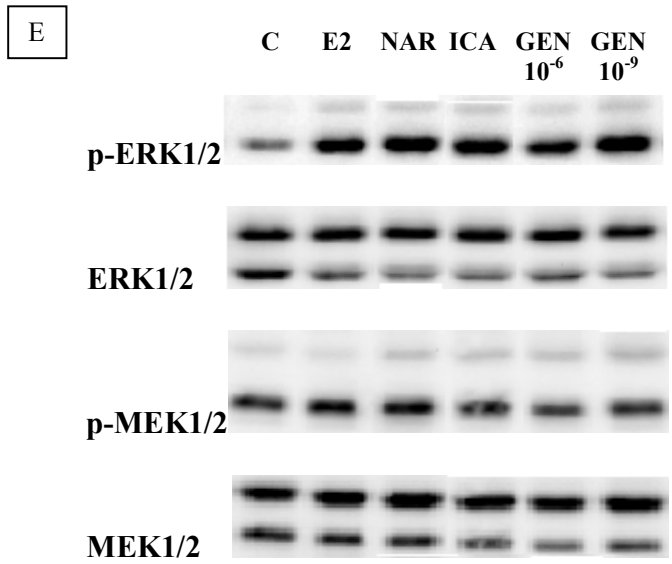


Figure 4.6: Effects of Naringin, Icariin and Genistein on phosphorylation of ERK and MEK in UMR106 cells.

UMR106 cells were stimulated with vehicle, 17β-estradiol (E2, 10^{-8}M), **(A, C)** Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-7}M) or **(B, D)** Genistein (GEN, 10^{-9}M and 10^{-6}M) for 5, 10, 30 and 60 minutes and the cell lysates were collected. After SDS-PAGE, the blots were probed with anti-phospho-ERK1/2 (p-ERK), anti-ERK1/2, anti-phospho-MEK1/2 (p-MEK) and anti-MEK1/2 primary antibodies. **(E)** Representative blots of 10 minutes treatment to phosphorylation of ERK and MEK. The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from two individual experiments (n=4) and expressed as mean $\pm$ SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

4.3.5 Effects of flavonoids on PI3K/Akt Pathway in UMR106 cells

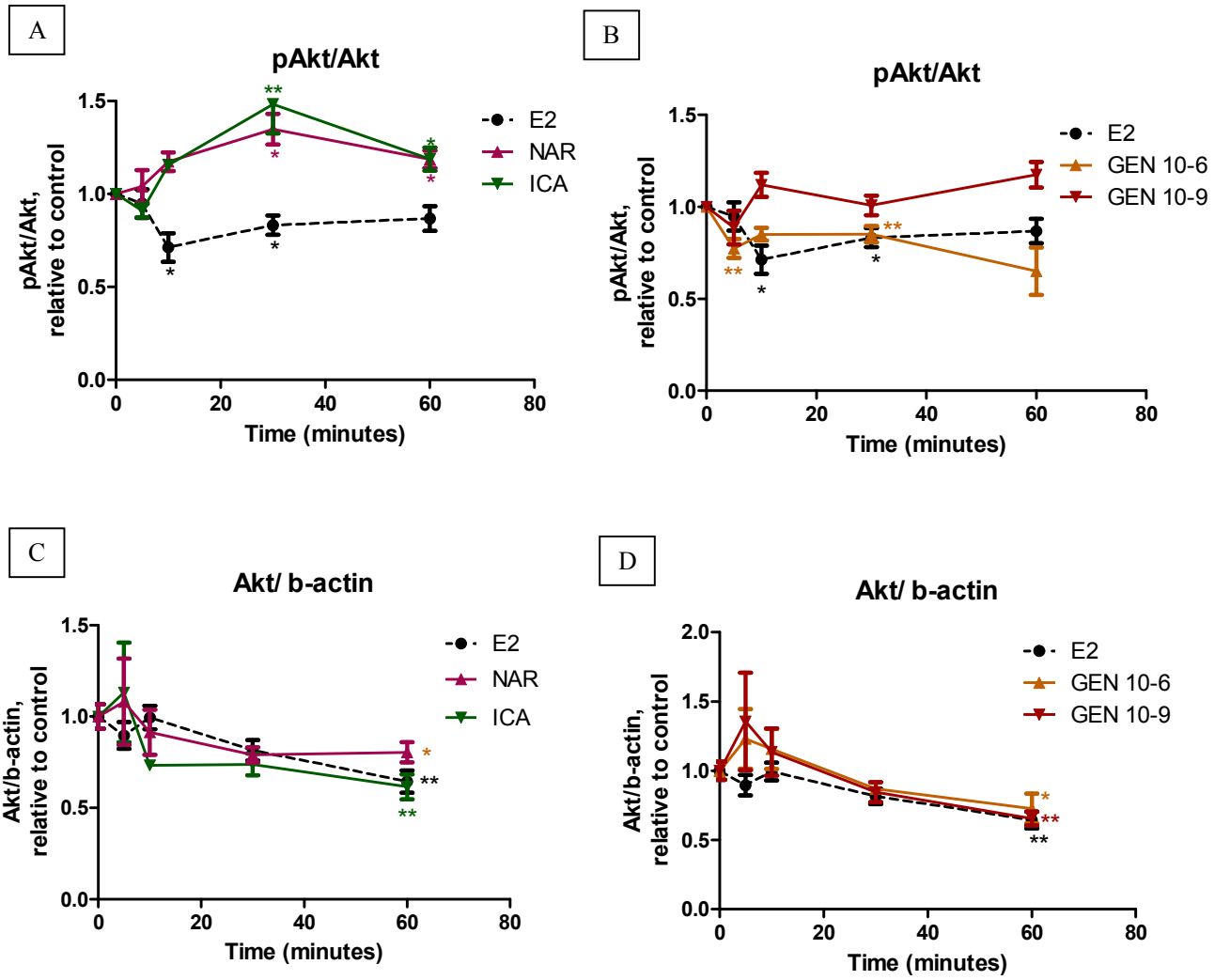
To assess the abilities of the flavonoids to induce activation of PI3K/Akt signaling cascades, the effects of flavonoids on phosphorylation of Akt and expression of PI3K and Akt in UMR106 cells were investigated. The cells were treated with E2 (10^{-8}M), naringin (10^{-7}M), icariin (10^{-7}M) and genistein (10^{-6}M, 10^{-9}M) at different time points (5mins, 10mins, 30mins, 60mins) and their effects on Akt phosphorylation and expression of Aktand PI3K were assessed by immunoblotting and normalized with β-actin.

The result showed that treatment with naringin and icariin for 30 minutes in UMR106 cells induced phosphorylation of Akt for around 1.35-fold ($P<0.05$) and 1.48-fold ($P<0.01$) and the activation sustained for an hour. In contrast, treatment with E2 and 10^{-6}M genistein led to decrease in Akt phosphorylation at 5 minutes to 30 minutes in UMR 106 cells. At 30 minutes, both E2 and 10^{-6}M genistein decreased the pAkt/Akt ratio for around 0.8-fold ($P<0.05$).10^{-9}M genistein did not alter Akt phosphorylation in UMR 106 cells from 5 minutes to 1 hour.

The protein expressions of Akt and PI3K in UMR 106 cells in response to E2 as well as flavonoids treatment were found to be unchanged during the short term treatment (5, 10 and 30 minutes), but a decreasing trend was observed after an hour. Treatment with estradiol for an hour led to decreased level of Akt expression for around 0.6-fold ($P<0.01$) and PI3K expression for around 0.7-fold ($P<0.05$) in UMR 106 cells. Treatment of cells with naringin and icariin led to a 0.8-fold ($P<0.05$) and 0.64-fold ($P<0.01$) decrease in Akt expression, respectively, as well as 0.6-fold ($P<0.01$) and 0.55-fold ($P<0.05$) decrease in PI3K expression after an hour treatment. Treatment with 10^{-9}M and 10^{-6}M genistein for an hour also led to a significant decrease in Akt expression for around 0.7-

fold and PI3K expression for around 0.6-fold in UMR 106 cells.

The results showed that naringin and icariin rapidly activated the PI3K/Akt Pathway via increasing the phosphorylation of Akt. Such results were opposite to the actions of estradiol and genistein in UMR106 cells which demonstrated an attenuated Akt phosphorylation upon treatment. E2 as well as all the flavonoids tested could significantly down-regulate the protein expression of Akt and PI3K when treated for an hour.



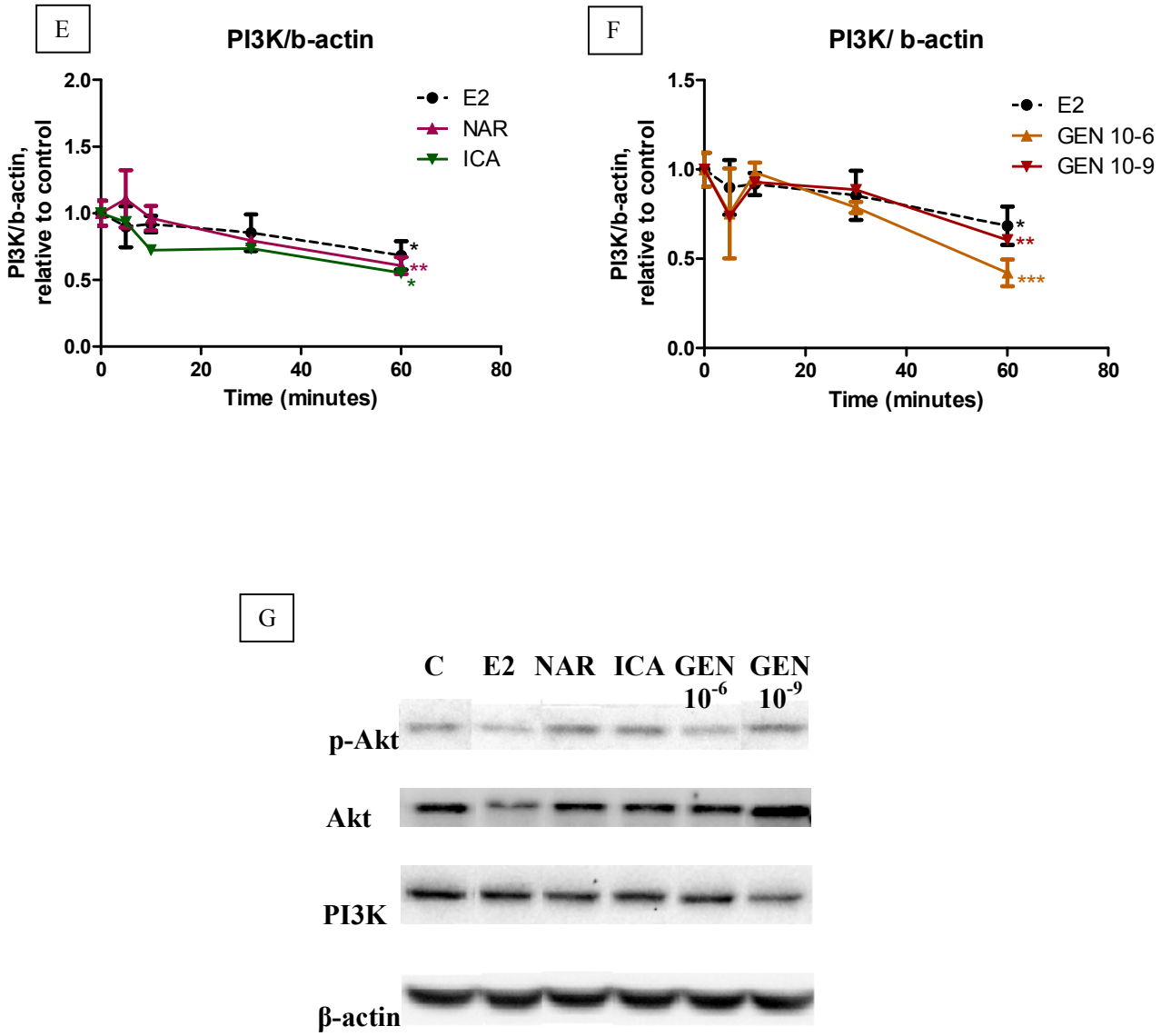


Figure 4.7: Effects of Naringin, Icariin and Genistein on phosphorylation of Akt and expression of Akt and PI3K in UMR106 cells.

UMR106 cells were stimulated with vehicle, 17β-estradiol (E2, 10^{-8}M), **(A, C, E)** Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-7}M) or **(B, D, F)** Genistein (GEN, 10^{-9}M and 10^{-6}M) for 5, 10, 30 and 60 minutes and the cell lysates were collected. After SDS-PAGE, the blots were probed with anti-phospho-Akt (pAkt), anti-Akt1/2/3 (Akt), anti-PI3K p85 subunit (PI3K) and anti-β-actin primary antibodies. **(G)** Representative blots of 30 minutes treatment to phosphorylation of Akt, expression of total Akt, PI3K and β-actin. The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from two individual experiments (n=4) and expressed as mean $\pm$ SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

4.3.6 Actions of flavonoids on cAMP-mediated signaling

Effects of flavonoids on intracellular cAMP accumulation

After 24 hours incubation in steroid-starved condition, UMR106 cells were treated with vehicle, PTH (10^{-8}M), 17β-estradiol (10^{-8}M), Naringin (10^{-7}M), Icariin (10^{-7}M) or Genistein (10^{-9}M) for 10 minutes and the cell lysates were collected for the measurement of intracellular cAMP.

Based on the result, treatment with PTH for only 10 minutes induced a substantial and rapid boost in intracellular cAMP level (80-fold increase, $P<0.001$) in UMR 106 cells. To our surprise, treatment of cells with E2 for 10 minutes not only fail to increase the intracellular cAMP content as predicted, but actually resulted in a significant drop in the cAMP level than control (0.72-fold compare to control, $P<0.05$).

For the phytoestrogens, a 0.77-fold decrease of intracellular cAMP content in UMR 106 cells ($P<0.05$) was recorded when the cells were treated with naringin. Icariin and genistein, on the other hand, showed no significant changes in cAMP level in UMR 106 cells after treatment for 10 minutes.

The results showed that 17β-estradiol as well as the flavonoids did not signal via induction of intracellular cAMP accumulation in rat osteoblastic UMR106 cells.

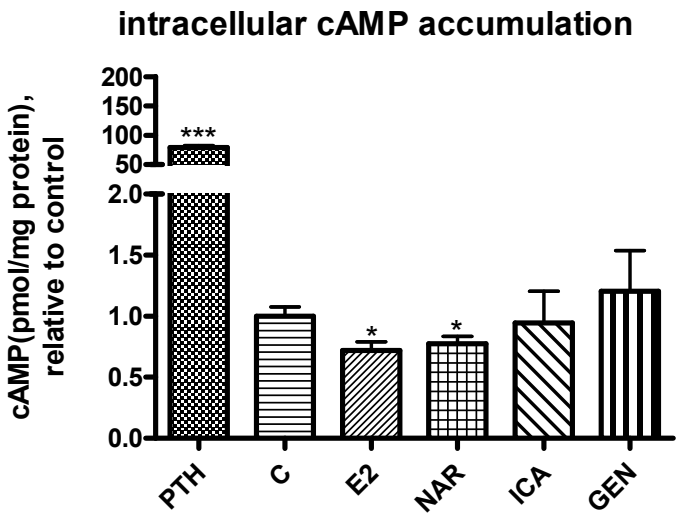


Figure 4.8: Effects of PTH, estrogen, naringin, icariin and genistein on intracellular cAMP production in UMR106 cells.

UMR106 cells were treated with vehicle(C), Parathyroid hormone (PTH,10^{-8}M), 17β-estradiol (E2,10^{-8}M), Naringin(NAR,10^{-7}M), Icariin(ICA,10^{-7}M) or Genisten(GEN, 10^{-9}M) for 10 minutes. Cell lysates were collected at the end of experiment and intracellular cAMP accumulation were deteceted by ELISA kit. Relative cAMP concentration was shown as fold over vehicle control and normalized with the total protein content measured by Bradford assay. Results were obtained from 2 individual experiments (n=4) and expressed as mean $\pm$ SEM.

* $P<0.05$;***$P<0.001$ versus vehicle control.

Effects of flavonoids on CRE-dependent luciferase activity

To confirm the results of intracellular cAMP assay, the abilities of naringin, icariin and genistein to induce transcriptional activities of CRE promoter-containing luciferase were investigated. Consistent with the intracellular cAMP results, treatment of UMR 106 cells with the positive control PTH (10^{-8}M) for only 10 minutes was sufficient to induce the CRE-dependent transcription to 2.67-fold ($P<0.01$vs control). On the other hand, 17β-estradiol failed to stimulate CRE-dependent luciferase activity in UMR 106 cells at most of the concentration tested (10^{-10}M to 10^{-7}M) except for 10^{-6}M, which induced a 1.4-fold increase ($P<0.001$) in luciferase activity. Naringin and icariin at all concentrations tested (10^{-6}M to 10^{-9}M) were unable to stimulate CRE-dependent luciferase activity in UMR106 cells. Treatment with genistein at all concentrations tested not only failed to induce CRE transcriptional activitiy, but also led to suppression of the CRE-luciferase activity. Specifically, CRE-luciferase activities were suppressed to 0.68-fold ($P<0.01$) and 0.88-fold ($P<0.05$) when UMR 106 cells were treated with 10^{-10}M and 10^{-9}M genistein, respectively.

Together with the intracellular cAMP assay, the results indicated that the rapid cellular signaling of flavonoids as well as estradiol in UMR106 cells did not involve the increase in intracellular cAMP production and the subsequent activation of CRE-dependent transcriptional events.

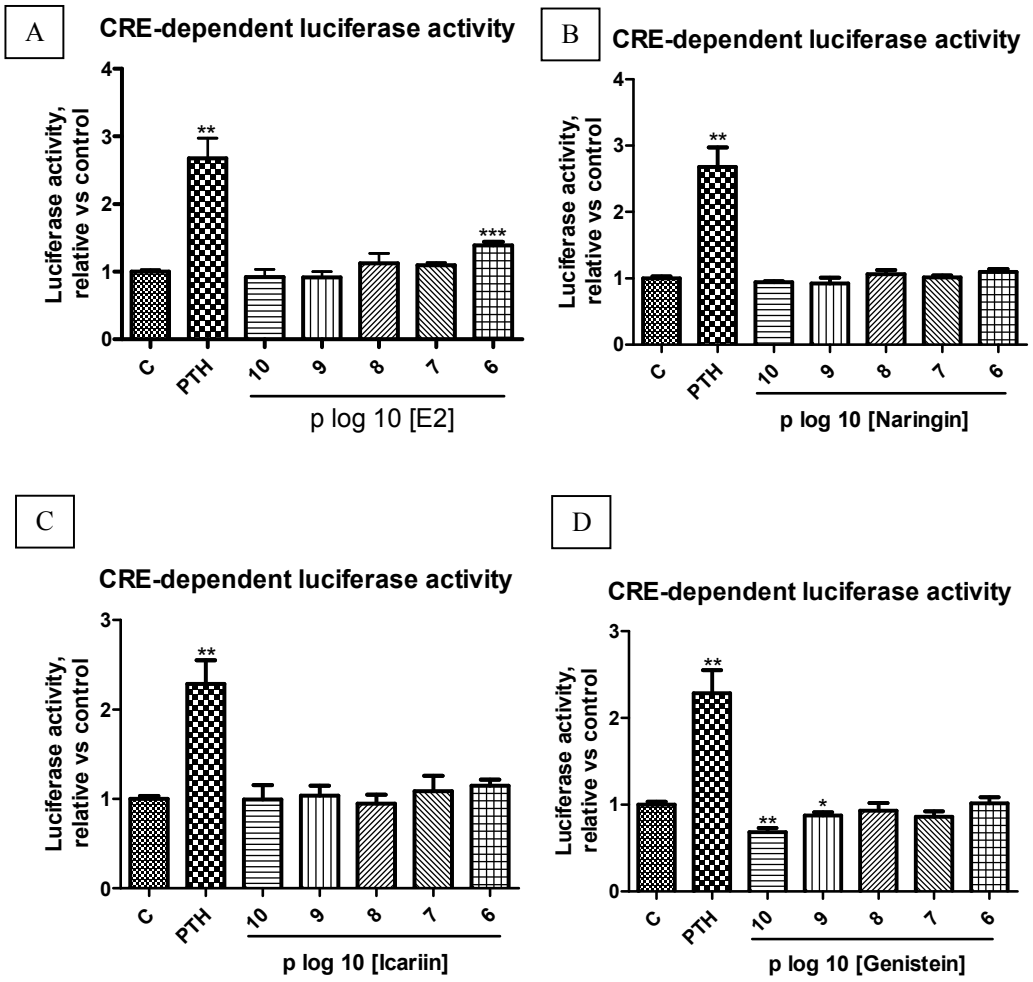


Figure 4.9: Effects of treatment with E2 (A), Naringin (B), Icariin (C), and Genistein (D) on inducing CRE-dependent luciferase activity in UMR106 cells.

UMR106 cells were co-transfected with 0.25ug pCRE-luc and 0.01ug pRL-TK with Lipofectamine 2000 and incubated for 6 hours. The cells were subjected to treatments with vehicle (C), parathyroid hormone (PTH, 10^{-8}M), E2, naringin, icariin or genistein (10^{-10}M to 10^{-6}M) for 10 minutes. Firefly and Renilla Luciferase activities were measured sequentially with Dual Luciferase Assay reagents. Results were obtained from 2 individual experiments (n=4) and expressed as mean $\pm$ SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

4.3 Discussions

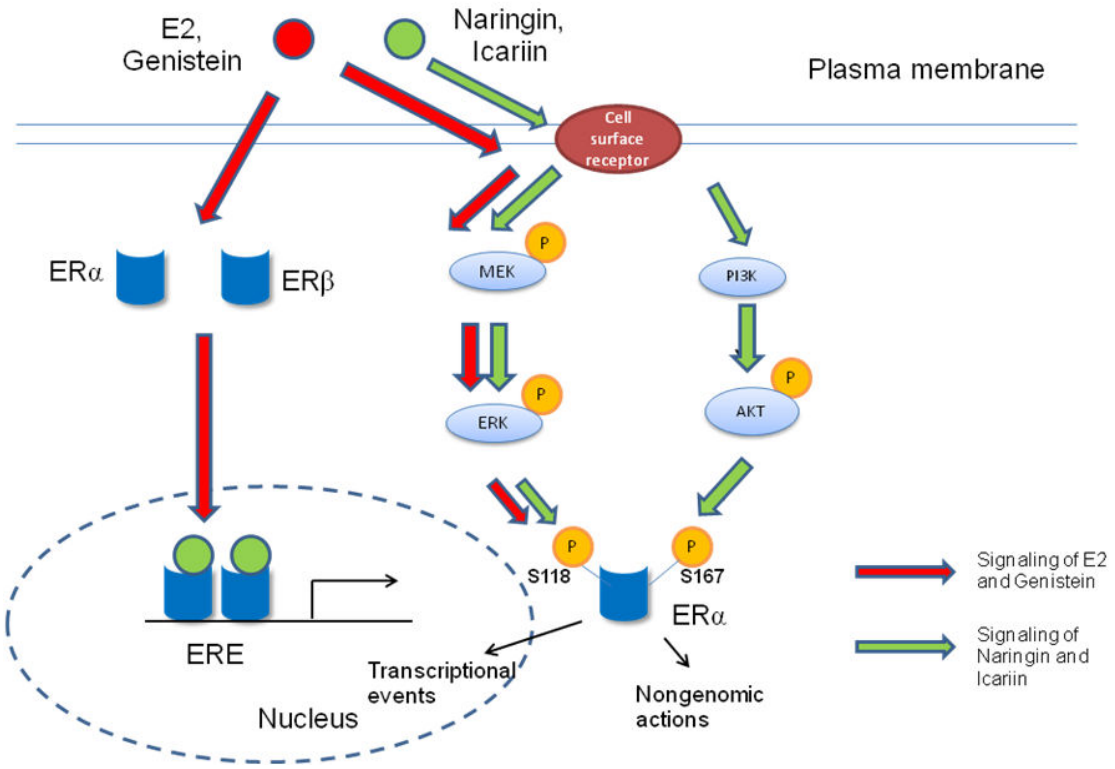


Figure 4.10: Proposed model for the signaling mechanisms of E2 and flavonoids in UMR106 cells.

E2 and Genistein stimulate classical genomic E2 signaling and activate MAPK pathway, while the actions of naringin and icariin are independent of classical E2 pathway and activate both MAPK pathway and PI3K/Akt signaling. The flavonoids induced the rapid phosphorylation of ERK which in turn phosphorylate ERα at Serine 118, while it is proposed that rapid activation of PI3K/Akt by Naringin and Icariin will also lead to ligand-independent phosphorylation of ERα at a different site (Serine 167).

Our previous (Mok et al. 2010; Pang et al. 2010) and current findings clearly

demonstrated that naringin, icariin and genistein exert positive effects in bone *in vivo* as well as *in vitro* using rat-osteoblastic UMR106 cells. However, the signaling mechanisms in which the flavonoid compounds exert their estrogen-like effects, particularly in osteoblasts, remained unknown. In the present study, the possible signaling routes of each flavonoid via classical genomic signaling and rapid estrogenic signaling cascades which involved activation of kinases and secondary messenger molecules were systematically evaluated.

To investigate if the flavonoids signal via classical genomic pathway, we examined the ability of each compound to bind directly to the classical estrogen receptors ERα and ERβ to activate them and induce transcriptional activity mediated by ERE promoter. The competitive binding results showed that among the flavonoids, genistein was the only one that was able to bind both ERα and ERβ. Naringin and icariin failed to displace more than 10% of the radioactive [^{3}H]-E2 in binding to ERs even at concentration 1000-fold higher than the latter and thus were regarded as non-binders by definition (Matthews et al. 2000).

The results demonstrated a close relationship between the ER binding abilities of flavonoids with their structures. It was previously reported that the glycosides bound poorly to the ERs, probably due to steric hindrance of the side groups with the binding domain of the receptors. (Morito et al. 2001) The study found that daidzin, glycitin and genistin demonstrated poor binding affinities with both ERs in compare with their respective aglycones. In this study, we demonstrated that naringin and icariin, as the glycosides of naringenin and icaritin respectively, could not directly bind to the ligand binding domain of estrogen receptors. On the other hand, naringenin had been shown to bind weakly to the estrogen receptors (Kuiper et al. 1998) while to our knowledge, no

study has been performed by date to investigate the binding affinity of icaritin. The results of genistein are consistent with the findings of others showing genistein bound human ERβ with a greater affinity than ERα (Kuiper et al. 1997; Morito et al. 2001).

To assess if the ligand binding affinity to ERs affects the genomic estrogenic pathway in osteoblasts, UMR106 cells were transiently transfected with ERE-luciferase reporter plasmid together with plasmids encoding ERα or ERβ before treatment. The plasmids encoding human ERα and ERβ were obtained from Dr Vincent Giguere and widely utilized in many of our previous studies in MCF-7 cells as well as in UMR-106 cells. It is known that ER is highly conserved among species and previous study reveals that there are 88% homologies between the sequences of human ERα/β with their rat counterparts, making the use of human ER in this cell line practicable. In addition, their DNA binding domains are nearly identical (93~ 96%) and the phosphorylation sites in AF-1 domain of ERα including Ser118 are conserved between species (Koike et al. 1987).

The effects of flavonoids in regulating ERE-dependent transcription via ERα and ERβ agreed with the binding affinity study of the flavonoids to estrogen receptors. In the current study, we found that naringin and icariin at most concentrations tested were unable to stimulate ERE-luciferase activity in UMR106 cells either transiently transfected with ERα or ERβ. Genistein, on the other hand, was potent in inducing both ERα and ERβ-mediated ERE-luciferase activities, although the activation of ERβ induced a greater ERE-dependent transcriptional activity than ERα. The selectivity of ERβ over ERα on ERE transcription for genistein could be the direct result of its greater affinity to bind ERβ.

Taken together, in the context of classical estrogen signaling pathway, the current

study demonstrated that naringin and icariin, despite their promising potency in improving bone properties, showed no binding affinity to both ERα and ERβ and could not induce ERE-mediated transcriptional activity on UMR106 cells via both ERs at various concentrations tested. The estrogenic effects of genistein, on the other hand, appeared to be a result of direct interaction with ERα and ERβ and therefore trigger the classical estrogen signaling which involve binding of ligand-bound ERs to ERE promoter region.

Our findings on anabolic effects of flavonids in UMR106 cells demonstrated that the effects of the flavonoids were ER-dependent as they could be abolished by ER specific antagonist ICI182, 780. Since our results revealed that the actions of naringin and icariin were independent of classical ERE-mediated genomic pathway, we hypothesized that these flavonoids might activate ERα in a ligand-independent manner. The results showed that naringin, icariin and genistein could indeed activate ERα via enhancing the phosphorylation of serine 118 residues in the AF-1 domain of ERα, which was consistent with the previous findings of our group (Mok et al. 2010; Pang et al. 2010). As mentioned, Ser 118 residues of ERα could be phosphorylated by either estrogen binding or extracellular signals such as growth factors. The phosphorylation of ERα induced by genistein might be due to its estrogen-mimicking properties in direct binding to the ERs. Besides, previous findings of our group in MCF7 cells suggested that genistein might enhance cross-talk between IGF-1R and ER and lead to the downstream phosphorylation mediated by the growth factor receptors (Chen et al. 2004). In the case of naringin and icariin, since no binding affinities to ERs were detected, we hypothesized that these phytoestrogens might activate the ERα via indirect mechanisms in UMR106

cells.

It was reported that the ligand-independent activation of ERα could be triggered by a variety of extracellular signals including the growth factors EGF and IGF-1 by making use of the rapid cellular signaling cascades including MAPK signaling (Kato et al. 1995; Bunone et al. 1996), PI3K/Akt signaling (Campbell et al. 2001) and intracellular cAMP-mediated signaling (Ince et al. 1994). This study focused on these 3 rapid signaling pathways and we have investigated the actions of flavonoids via each of the possible pathway in UMR 106 cells.

MAPK signaling, in particular, had been shown to directly involve in the functional activation of unliganded ERα via the phosphorylation of serine 118 residues (Chen et al. 2002) as well as mediating the nongenomic signaling of E2 in bone cells without inducing transcriptional activity (Kousteni et al. 2001). As the activation of MAPK signaling by extracellular signals occurred in a rapid time frame, we monitored the level of phosphorylation of MEK1/2 and ERK1/2 within an hour in response to flavonoids treatments. Here we demonstrated that naringin, icariin and genistein could stimulate the phosphorylation of ERK1/2 as rapid as 5 minutes and the degrees of stimulation within the first 10 minutes were even greater than E2 itself. Genistein were administered at a high concentration (10^{-6}M) and a low concentration (10^{-9}M) to observe if its role as a tyrosine kinase inhibitor at higher concentration would influence its action on MAPK signaling. The results showed that genistein was still potent in activating ERK1/2 at the concentration of 10^{-6}M. Moreover, their signals via phosphorylation of ERK1/2 had shown to sustain for at a level comparable to E2 for at least an hour, which might contribute to the sustained phosphorylation of ERα at Ser118 when the cells were

incubated for 24 hours under these flavonoid compounds. The degree of MEK1/2 phosphorylation, on the other hand, remained unchanged upon treatment with E2 as well as the phytoestrogens.

PI3K/Akt pathway is another important pathway which regulates the estrogen-independent activation of ERα (Campbell et al. 2001) and promotes cell survival in a wide variety of cell types (Datta et al. 1999).Our findings on PI3K/Akt pathway, in particular on the phosphorylation of Akt at Ser493, showed different regulation by naringin and icariin in comparison to E2 and genistein. Naringin and icariin were able to induce Akt phosphorylation in UMR106 cells as early as 30 minutes, while treatment with E2 and genistein (10^{-6}M) inhibited the basal activation of Akt. All compounds tested down-regulated the protein expression of Akt and PI3K in UMR 106 cells within an hour.

Our study showed that E2 seemed to decrease the basal Akt activation in UMR 106 cells. This observation is in agreement with those reported by Sunters et al in which E2 tended to decrease the basal Akt activation while IGF-I stimulated phosphorylation of Akt (Sunters et al. 2010). This is in sharp contrast to the reported stimulatory effects of E2 on Akt activity in other cell line such as breast cancer cells (Ahmad et al. 1999) and neuronal cells (Honda et al. 2000). The results indicate that the regulation of PI3K/Akt pathway by E2 is indeed cell-type specific. Genistein at a high concentration 10^{-6}M were also shown to inhibit Akt phosphorylation as well as protein expression of Akt and PI3K in UMR 106 cells. Such action might be associated with its role as tyrosine kinase inhibitors or its estrogen-like effects in UMR106 cells. On the other hand, naringin has been demonstrated to enhance Akt phosphorylation to induce BMP2 production in MC3T3-E1 cells (Wu et al. 2008). Signaling of icariin via PI3K/Akt pathway in

osteoblastic cells was not well studied, but it has been reported to activate Akt in RAW264.7 macrophage (Xu et al. 2010). The enhancement of Akt phosphorylation by naringin and icariin in UMR106 cells as shown in the present study might serve as signals distinct from E2 signaling to promote osteoblastic cell survival and proliferation.

The abilities of estrogens and phytoestrogens to rapidly regulate intracellular cAMP accumulation and its downstream target CRE-promoter were also investigated. PTH was used in this study as positive control due to the high responsiveness of UMR106 cells to this hormone. Treatment of UMR106 cells with PTH were shown to rapidly produce a substantial amount of cAMP due to high abundance of PTH receptor on this cell line (Ahlström et al. 1999).

The result of PTH was consistent with findings by others (Ahlström et al. 1997) in which some 85-fold increase of intracellular cAMP level were found in UMR 106 cells in response to 10 minutes of PTH treatment. However, to our surprise, treatment with estrogen and our tested compounds in UMR106 cells failed to induce the rapid intracellular cAMP accumulation like they did on breast cancer cell lines. On the contrary, estrogen was found to decrease the cAMP level by around 30% in UMR 106 cells. The similar inhibition of cAMP accumulation was also found in naringin-treated group which recorded a 23% decrease in intracellular cAMP level. The results were further confirmed by the CRE-dependent luciferase activity assay, which shown no induction of the transcriptional activity in UMR 106 cells upon treatment with E2 as well as the flavonoid compounds. To our knowledge, this is the first study to investigate the direct effect of E2 on regulating intracellular cAMP level in osteoblasts. However, there were some indirect findings which could suggest some possible reasons for this estrogenic response. It was

found that in SaOS-2 osteoblastic cells, pre-treatment with E2 inhibited by up to 50% of the accumulation of cAMP stimulated by hPTH/ hPTHrP (Fukuyama et al. 1989). E2 was also found to suppress cAMP-activated IGF-1 expression (McCarthy et al. 1997). Furthermore, cAMP accumulation in osteoblastic cells was found to inhibit proliferation, ALP activity and expression of bone formation markers such as Runx2, OCN and OPN. Thus, it was obvious that the effect of estrogen treatment on intracellular cAMP accumulation was cell type-dependent and significant differences between the responses of breast cancer cells and osteoblasts were found.

In summary, we found that the distinct mechanisms might be involved in actions of naringin, icariin and genistein in UMR106 cells. The estrogenic actions of genistein were found to be more similar to E2 in stimulating classical estrogen signaling and regulating MAPK and PI3K/Akt pathway, while the actions of naringin and icariin might be different from E2 in the way that they did not induce classical estrogen signaling and regulate PI3K/Akt signaling differently. Nonetheless, all three flavonoids were able to stimulate the ligand-independent activation of ERα and the rapid MAPK signaling via phosphorylation of ERK.

Chapter 5

Role of GPR30 in the estrogenic signaling of phytoestrogens in UMR106 cells

5.1 Introduction

It was suggested by some studies (Kelly et al. 2001; Razandi et al. 2003) that classical ERs were responsible for the rapid cellular signaling and ERK1/2 activation. However, the lack of functional motifs in ERα and ERβ for non-genomic mechanism in estrogenic signaling and the occurrence of some estrogenic responses in cells lacking classical ERs lead to speculation that another protein(s) might be involved (Filardo et al. 2000). Interestingly, GPR30-dependent rapid cellular signaling is known to mediate the downstream kinases regulated by E2 as well (Filardo et al. 2002; Prossnitz et al. 2008). It was proposed that estrogen, when binding to GPR30 receptor on membrane, promotes EGFR trans-activation via release of heparin-bound EGF (HB-EGF) and stimulates ERK1/2 activity through downstream signaling cascade **(Fig 5.1)**. ERK1/2 phosphorylation, in turn, activates ER and the transcriptional activity. This suggests that non-genomic pathways in which the phytoestrogens exert their effects might be mediated by the activation of GPR30 and possible trans-activation of ER by the mean of ligand binding to GPR30 that trigger the signaling events.

In light of this, we speculated that the nongenomic signaling pathways exerted by estrogen and the flavonoids in UMR106 cells might be exerted by the following steps: 1) Apart from binding directly to ER, estrogen and the flavonoids directly/indirectly activates GPR30; 2) the activated GPR30 induce a series of rapid cellular signaling events that lead to phosphorylation of MAPK or Akt; 3) the activated MAPK or Akt in turn phosphorylates and activates ERα; 4) Activated ERα induces the tissue responses including cell proliferation and differentiation. Hence, to find out the possible involvement and molecular mechanisms of GPR30-mediated signaling in the

phytoestrogens-induced effects in osteoblasts, the specific antagonist of GPR30, G15 were pre-incubated in UMR106 cells prior to the flavonoids treatment to observe if the blocking of GPR30 activation would lead to alteration of these estrogenic responses exerted by the flavonoids as well as E2. In addition, the functional effects of specific agonist of GPR30, G1 on UMR106 cells proliferation and differentiation were studied.

We proposed that GPR30-mediated signaling might be involved in modulation of nuclear transcriptional activities of ERE promoter-containing genes, which are the target genes for classical ER-mediated genomic signaling as well. The abilities of phytoestrogens to activate each ER isotype and stimulate the transcriptional activity of ERE-luciferase gene were demonstrated in the last chapter. To determine if GPR30 is indeed involved in this event, the transfected UMR106 cells were pre-incubated with the GPR30 specific antagonist, G15 prior to treatment to observe its ability to antagonize the phytoestrogen-induced ERE-dependent luciferase activity.

In the approach to investigate the effects of phytoestrogens on the accumulation of intracellular cAMP in UMR-106 cells, we found that treatment with estrogen and the flavonoid compounds did not lead to any rise in the cAMP level. In contrast, PTH treatment induced a rapid increase in the cAMP while treatment with E2 and naringin even led to reduction in the intracellular cAMP to a level lower than vehicle treatment. To investigate the involvement of GPR30 in the regulation of intracellular cAMP concentration by these compounds, the effects of pre-treatment with G15 on cAMP concentration were examined.

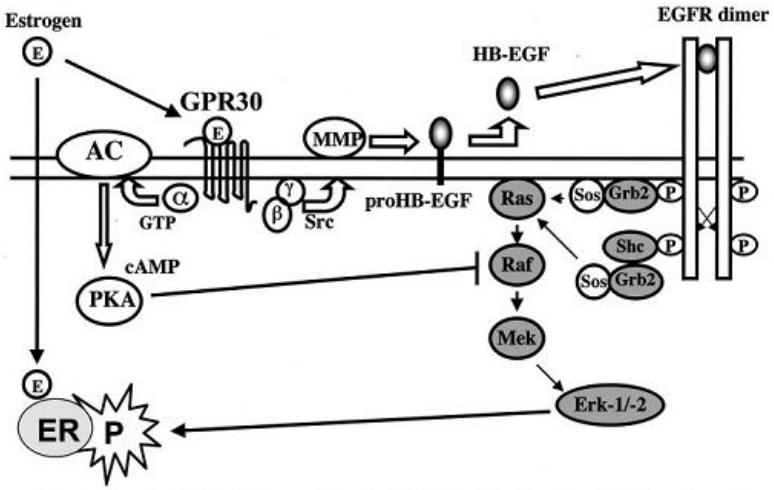


Fig. 10. Proposed Mechanism by Which Estrogen Acts via GPR30 to Regulate Growth Factor Receptor and ER Signal Transduction Pathways

Data presented here suggest that via GPR30, estrogens as well as antiestrogens are capable of stimulating adenylyl cyclase activity, which in turn, leads to PKA-mediated suppression of EGF-induced Erk-1/-2 activity. Previously, we have shown that estrogen and antiestrogens act via GPR30 to promote EGFR transactivation through a G$\beta\gamma$-subunit protein pathway that promotes Src-mediated, metalloproteinase (MMP)-dependent cleavage and release of HB-EGF from the cell surface. Thus, via GPR30, estrogen may balance Erk-1/-2 activity by stimulating two distinct G protein signaling pathways that have opposing effects on the EGFR-to-MAPK axis.

Figure 5.1: Proposed Mechanism of estrogen action via GPR30 (Filardo et al. 2002)

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5.2 Methodology

5.2.1 Cell Culture

Rat osteoblastic UMR106 cells: The rat osteoblastic UMR 106 cells (ATCC® Number: CRL-1661™**)** were cultured as described previously in 3.2.1.

5.2.2 Cell Proliferation and Differentiation Assay

The cells were seeded in 96-well plate. After 24 hours incubation in steroid-starved medium, vehicle, E2 (10^{-8}M) and G1 (10^{-12}M to 10^{-5}M) (Tocris Bioscience, USA) were administered. MTS assay and ALP assay were performed as described previously.

5.2.3 ER -mediated ERE-dependent luciferase activity assay

The ERE-luciferase activity assays were performed as described previously in 4.2.2 with minor modification. Briefly, 6 hours after transfection, vehicle, estradiol (10^{-8}M), naringin (10^{-10}M to 10^{-6}M) or genistein(10^{-10}M to 10^{-6}M) was added followed by 24 hours incubation in 37°C with or without pre-incubation of G15(10^{-6}M) 20 minutes prior to treatment. Then, the cells were lysed using PLB and Dual Luciferase Reporter Assay is carried out as described.

5.2.4 Immunoblotting

The cells were seeded in 6-well plate and immunoblotting were performed as described in 4.2.3. The blots were probed overnight at 4°C with the following primary antibodies: anti-human phospho-ERα at Ser118 (1:2000, Upstate), rabbit anti-human ERα (1:1000, Santa Cruz), rabbit anti-p85 PI3K (1:1000, Cell Signaling), rabbit anti-phsopho-Akt at Ser473 (1:1000, Cell Signaling), rabbit anti-Akt1/2/3 (1:1000, Santa Cruz), mouse anti-

phospo-ERK (1:1000, Santa Cruz), rabbit anti-ERK1/2 (1:1000, Cell Signaling), or mouse anti-β actin (1:2000, Abcam). The blots were then washed 3 times with TTBS followed by incubation with anti-rabbit (1:2000, Santa Cruz) or anti-mouse (1:2000, Cell Signaling Technology) IgG-HRP-conjugated secondary antibodies for 1-2 hours. ECL western blotting detection reagents (Clarity™ Western ECL Substrate, Bio-rad, USA) were added to the blots and the chemiluminescence of the bound antibodies were visualized and quantified by the Lumi-Imager F1 Workstation (Roche, Manheim, Germany). The intensity of the signal in the band was expressed as Biochemical Light Units (BLU).

5.2.5 Intracellular cAMP measurement

UMR106 cells were seeded in 6-well plate and incubated for 48 hours before changing to steroid-starved medium. After 24 hours incubation in phenol red free DMEM supplemented with sFBS, vehicle, PTH (10^{-8}M), estradiol (10^{-8}M) or naringin (10^{-7}M) were applied into the wells with or without 20 minutes pre-incubation with G15. After the drug stimulation for 10 minutes, the intracellular cAMP concentration was measured by using Cyclic AMP EIA Kit (Cayman, USA) following the methodology described previously in 4.2.4.

5.2.6 Statistical Analysis

Results were expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by two-tailed unpaired Student's t-test between the drug-treated group and vehicle-treated control group using Microsoft Office Excel 2007.A value of $p < 0.05$ was considered significant. All graphs in the study were plotted using GraphPad Prism Version 4.00

5.3 Results

5.3.1 Effects of GPR30 agonist, G1 in UMR106 cell proliferation and differentiation

As a GPR30 specific agonist, G1 treatment in UMR106 cell proliferation showed a biphasic result. At a lower concentrations (10^{-9}M and 10^{-8}M), G1 stimulated the growth of UMR106 cells. Treatment with 10^{-8}M G1 led to a 1.12-fold increase ($P<0.05$) in cell proliferation relative to control. However, the role of G1 switched from stimulatory to inhibitory at higher concentrations (10^{-6}M and10^{-5}M). Treatment of UMR cells with 10^{-5}M G1 resulted in a marked inhibition on cell growth, which was 0.67-fold ($P<0.001$) relative to control.

Treatment with G1 demonstrated either no effect or weak inhibitory effect on ALP activity of UMR106 cells. At certain concentrations (10^{-12}M, 10^{-9}M, 10^{-7}M, 10^{-6}M) a weak but significant ($P>0.05$) reduction in ALP activity was recorded after 24 hours incubation under G1 treatment. At other concentrations (10^{-11}M,10^{-10}M,10^{-8}M) G1 posed no obvious effect on cell differentiation in compare with control.

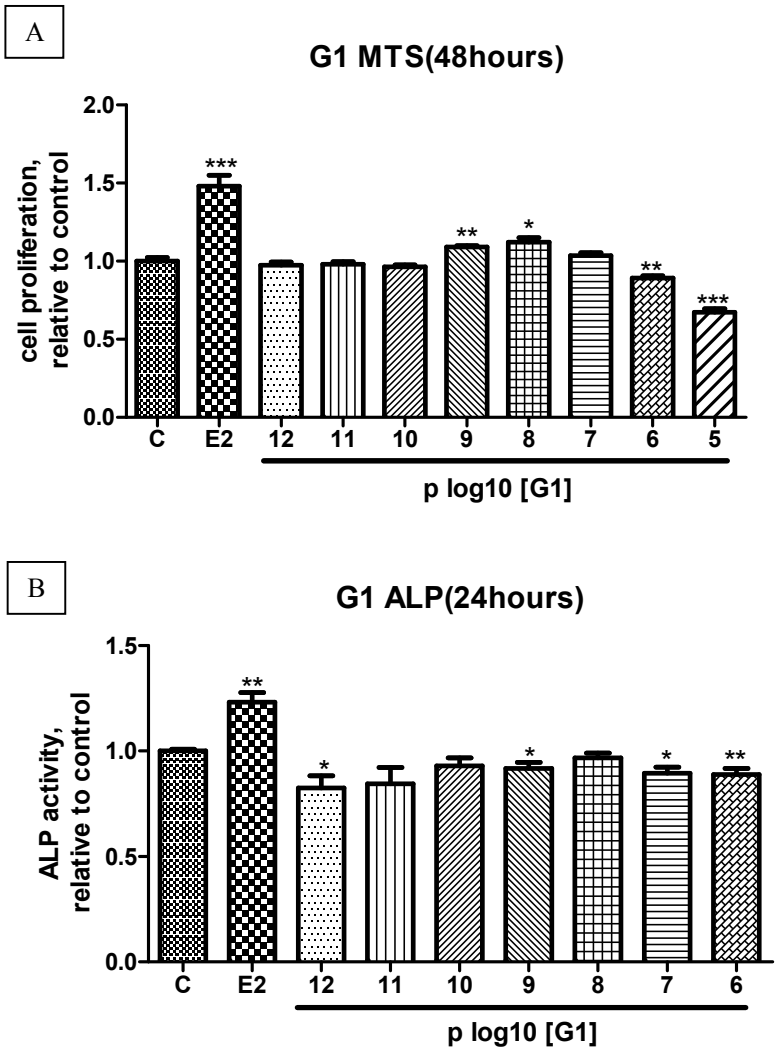


Figure 5.2: Effects of treatment with G1 on UMR106 cell proliferation and ALP activity. 5,500 cells/ well were cultured in 96-well microtiter plate and were subjected to drug treatments for 24/48 hours. **(A)** Cell proliferation rate was assessed by MTS assay. **(B)** Cell differentiation was indicated by ALP activity in the assay normalized with total protein content (OD_{595}) in Bradford assay. Cells were treated with vehicle(C), 17β-estradiol (E2, 10^{-8}M) or G1 (10^{-12}~10^{-5}M). Results were obtained from two experiments (n=6) and expressed as mean ± SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

5.3.2 Involvement of GPR30 and ER on genomic ER pathway

As the luciferase activity results of naringin and icariin showed no activity on inducing ERE transcription, only genistein at 10^{-6}M which demonstrated the highest induction among all the concentrations were further studied. To investigate the involvement of GPR30 in the signaling pathway that led to activation of ERE-dependent transcriptional activity, the transfected UMR106 cells were pre-incubated with 10^{-6}M G15 before treatment with estradiol (10^{-8}M), genistein (10^{-6}M) or vehicle for 24 hours. As a comparison, pre-incubation with ICI182, 780 (ICI) and co-incubation with both ICI and G15 were simultaneously studied to examine if they have any synergistic effects in modulating the ERE-mediated transcription.

The results showed that pre-treatment with ICI completely abolished the stimulation of ERE-dependent luciferase activity by E2 and genistein via both ERα and ERβ back to the basal level ($P<0.001$ vs treatment group without ICI). In fact, treatment with ICI itself had already led to a significant reduction of ERα-mediated luciferase signal on the basal luciferase activity (0.65-fold, $P<0.01$ vs control).

In contrast, pre-treatment with G15 could partially diminish the stimulation of ERα-mediated ERE-dependent luciferase activity by E2 and genistein ($P<0.001$), but did not affect the stimulation of ERβ-mediated ERE-dependent luciferase activity by either E2 or genistein. In UMR106 cells transfected with ERα, pre-treatment with G15 led to decrease in ERE-luciferase activity induced by E2 and genistein for 33% and 50%, respectively. Pre-treatment of UMR 106 cells with both G15 and ICI completely abolished the effects of E2 and genistein in ERE-luciferase activity via ERα and ERβ, Nonetheless, the antagonizing effects of pre-treatment with both G15 and ICI showed no

further reduction in comparison with the treatment group pre-incubated with ICI alone.

The results implied that GPR30 played a role in regulating estrogen and genistein-induced transcriptional events that involved the activation of ERE-promoter containing gene by ERα, but not ERβ. On the other hand, estrogen receptors alone were showed to be sufficient and most vital in modulating ERE-dependent transcription in UMR106 cells.

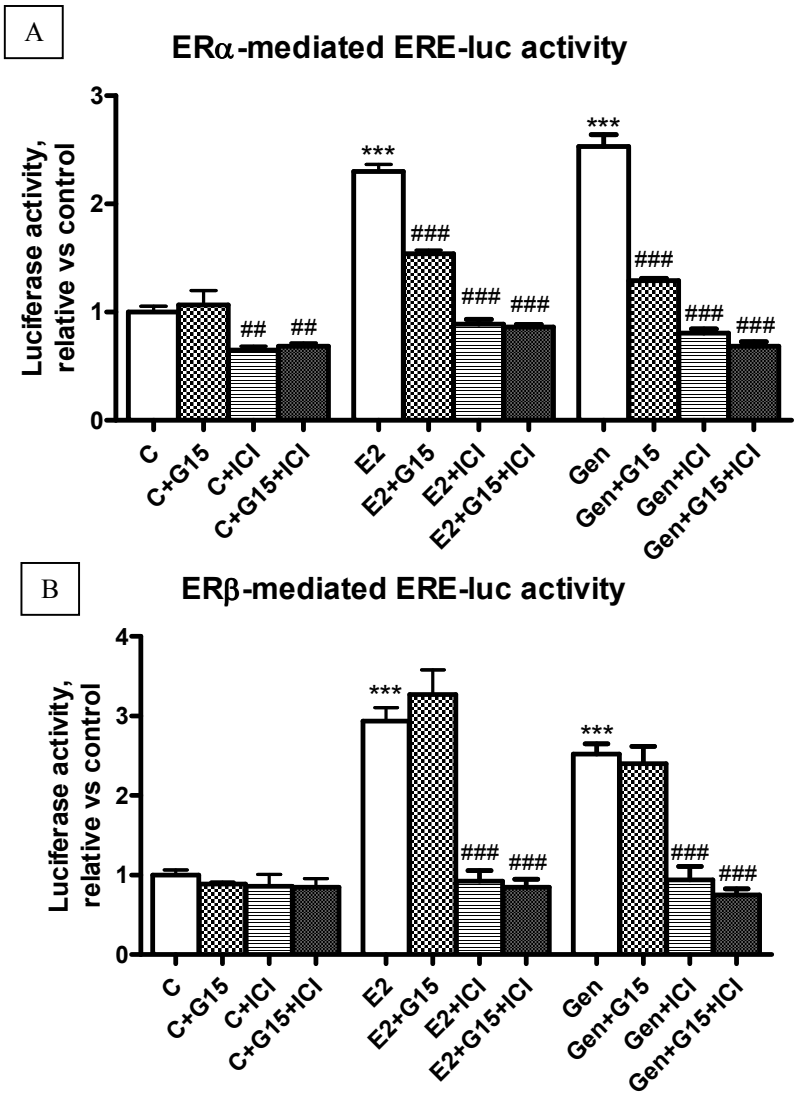


Figure 5.3: Effects of G15 on regulating estrogen and genistein-induced ERE-dependent luciferase activity in UMR106 cells.

UMR106 cells were cotransfected with 0.4ug ER-α or ER-β plasmid, 0.4ug vERETkluc, and 0.1ug pRL-TK with Lipofectamine 2000 and incubated for 6 hours. The cells were subjected to treatments with vehicle (C), 17β-estradiol (E2, 10^{-8}M) or Genisten (GEN, 10^{-6}M) for 24 hours in the presence or absence of G15 (10^{-6}M), ICI182, 780 (ICI, 10^{-6}M) or both G15 and ICI pre-incubation for 20 minutes prior to treatment. The cell lysates were collected and Firefly and Renilla Luciferase activities were measured sequentially with Dual Luciferase Assay reagents. The results were normalized with control and expressed as **(A)** ERα-mediated ERE-luciferase activity and **(B)** ERβ-mediated ERE-luciferase activity. Results were obtained from 2 individual experiments (n=4) and expressed as mean $\pm$ SEM.

* P<0.05;***P<0.001 versus control

#P<0.05 ; ##P<0.01###P<0.001 versus treatment group without antagonist addition.

5.3.3 Effects of G15 on phytoestrogen induced phosphorylation of ERα at Ser118

We have shown that E2 as well as all three flavonoids in our study could induce the activation of ERα in a ligand-independent manner. To determine if the ligand-independent actions of estrogen and the flavonoids to induce ERα phosphorylation at serine 118 residues are mediated by activation of GPR30, UMR106 cells were pre-incubated with G15 for 20 minutes prior to treatment to inactivate GPR30. Interestingly, our results showed that pre-treatment of UMR 106 cells with G15 not only failed to antagonize the effects of E2 and flavonoids, but even potentiated their effects in stimulating phosphorylation of ERα.

As shown in figure 5.4, treatment of UMR 106 cells with G15 alone were able to raise the basal phosphorylation of ERα at Ser118 to 1.8-fold higher than vehicle control ($P<0.05$). Moreover, pre-treatment of UMR 106 cells with G15 before treatment with E2 and flavonoids significantly increased the degree of phosphorylation of ERα than treatment with the compounds alone ($P<0.05$ vs control).

The results indicated that the ligand-independent activation of ERα in UMR 106 cells were not dependent on activation of GPR30, and G15 seemed to have an opposite effects.

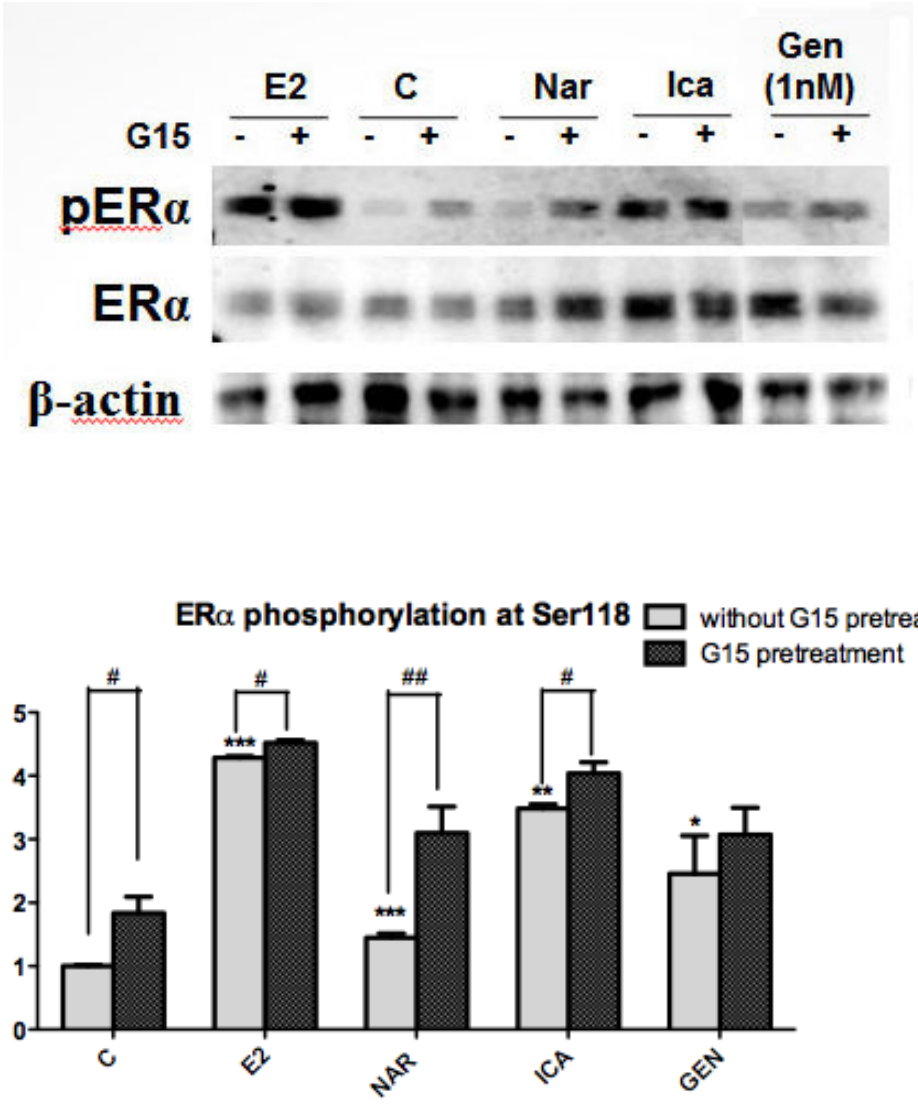


Figure 5.4: Effects of G15 on phosphorylation of ERα at Ser118 induced by E2 and flavonoids in UMR106 cells.

UMR106 cells seeded in 24 wells were co-transfected with 0.4ug ER-α/well with Lipofectamine 2000 and incubated for 6 hours. The cells were then subjected to treatments with vehicle (C), 17β-estradiol (E2, 10^{-8}M), Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-7}M) or Genistein (GEN, 10^{-9}M) for 24 hours with or without G15 pre-incubation for 20 minutes before treatment. The cell lysates were collected. After SDS-PAGE, the blots were probed with anti-phospho-ERα at Ser118 (pERα), anti-ERα and anti-β-actin primary antibodies. The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from three individual experiments (n=3) and expressed as mean $\pm$ SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

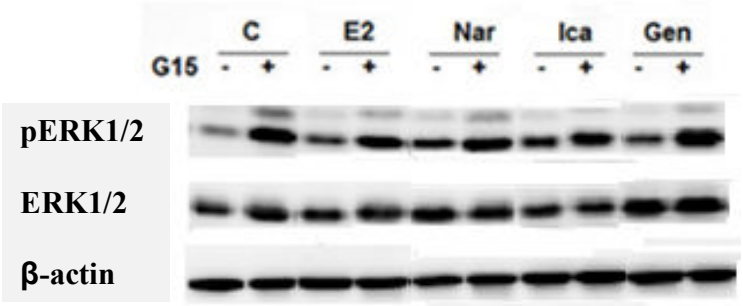
#P<0.05 ; ##P<0.01###P<0.001 versus treatment group without antagonist addition.

5.3.4 Effects of G15 on phytoestrogens induced rapid phosphorylation of ERK1/2 in UMR106 cells

To further confirm the effects of G15 on ERα phosphorylation at Ser118 and to explore the role of GPR30 in rapid cellular signaling system, we traced upstream to the rapid phosphorylation of ERK1/2 in UMR106 cells. We have shown that E2 as well as the flavonoids rapidly induce the phosphorylation of ERK1/2 and the responses were found to reach the peak at 10 minutes for most of the compounds. In this study, UMR106 cells were stimulated with E2 and the flavonoids for 10 minutes with or without pre-incubation with G15.

As shown in figure 5.5, treatment of UMR 106 cells with G15 alone significantly increased the basal phosphorylation level of ERK1/2 to 4.2-fold ($P<0.001$ vs. control). More importantly, when UMR 106 cells were pre-incubated with G15, the phosphorylation of ERK1/2 induced by E2 and the compounds were significantly higher than treatment without G15. The results demonstrated that pre-incubation of UMR 106 cells with G15 led to further increase in the pERK/ERK ratio for at least 2-fold ($P<0.05$) in each treatment group as compared to treatment with E2 or the flavonoids alone.

The results showed that G15 not only could not block the phosphorylation of ERK1/2 in UMR 106 cells, but even showed to have synergistic effects with E2 and flavonoids in promoting the phosphorylation of ERK1/2. Treatment of UMR 106 cells with G15 alone could also lead to increase in ERK1/2 activity.



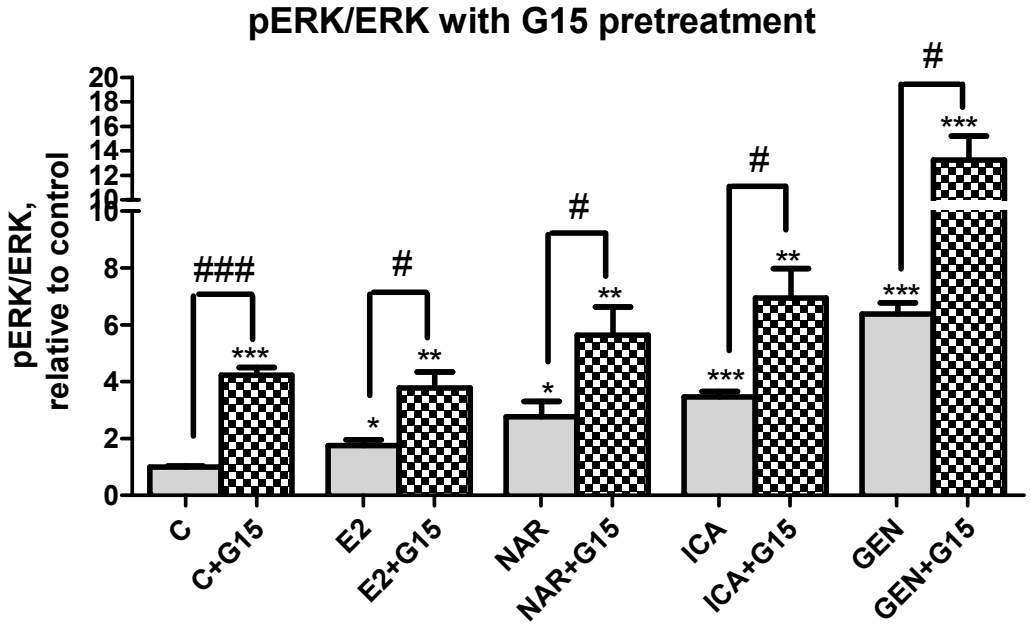


Figure 5.5: Effects of G15 on phosphorylation of ERK1/2 induced by E2 and flavonoids in UMR106 cells.

UMR106 cells were stimulated with vehicle (C), 17β-estradiol (E2, 10^{-8}M), Naringin (NAR, 10^{-7}M), Icariin (ICA, 10^{-7}M) or Genistein (GEN, 10^{-9}M) for 10 minutes with or without G15 pre-incubation for 20 minutes prior to treatment. The cell lysates were then collected and SDS-PAGE was performed. After transblotting to PVDF membrane, the blots were probed with anti-phospho-ERK1/2 (pERK) and anti-ERK1/2 primary antibodies. The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from two individual experiments (n=4) and expressed as mean $\pm$ SEM.

*P<0.05;**P<0.01;***P<0.001 versus control.

#P<0.05 ; ##P<0.01###P<0.001 versus treatment group without antagonist addition.

5.3.5 Effects of G15 on phosphorylation of Akt in UMR106 cells

In order to further investigate the role of GPR30 in the rapid estrogenic signaling cascades, we sought to find out the effects of G15 on the degree of phosphorylation of Akt in UMR106 cells regulated by E2 and the flavonoids. The cells were pre-incubated with G15 before treatment with E2 (10^{-6}M), naringin (10^{-7}M), icariin (10^{-7}M) and genistein (10^{-6}M) for 30 minutes.

In consistent with the previous results, treatment of UMR 106 cells with E2 and genistein showed decreased level of Akt phosphorylation compared to vehicle control, while naringin and icariin increased the phosphorylation to 1.3-fold and 1.18-fold, respectively ($P<0.05$, vs control). Interestingly, pre-treatment of UMR 106 cells with G15 led to an overall increased in Akt phosphorylation for each treatment group. In fact, the basal level of Akt phosphorylation in UMR 106 cells was increased to around 2.1-fold ($P<0.001$ vs control) in response to pre-treatment with G15 alone. Pre-incubation of UMR 106 cells with G15 effectively abrogated the inhibitory effects of E2 and genistein, and even led to 1.4-fold and 2.6-fold increase in Akt activity, respectively. The stimulation of Akt phosphorylation in UMR 106 cells by naringin and icariin were also further increased when the cells were pre-incubated with G15 prior to treatment.

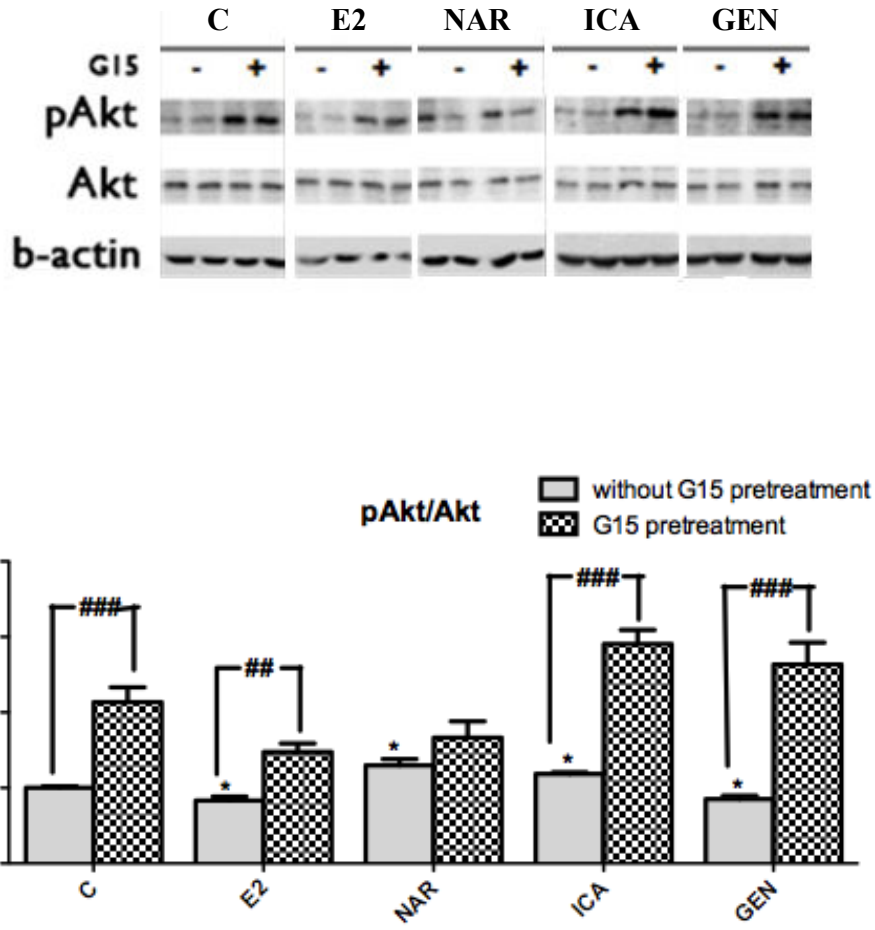


Figure 5.6: Effects of G15 on phosphorylation of Akt regulated by E2 and flavonoids in UMR106 cells.

UMR106 cells were stimulated with vehicle (C), 17β-estradiol (E2, 10^{-8}M), Naringin

(NAR, 10^{-7}M), Icariin (ICA, 10^{-7}M) or Genistein (GEN, 10^{-6}M) for 30 minutes with or without 20 minutes pre-incubation with G15 and the cell lysates were collected. After SDS-PAGE, the blots were probed with anti-phospho-Akt (pAkt), anti-Akt1/2/3 (Akt), anti-PI3K p85 subunit (PI3K) and anti-β-actin primary antibodies. The intensities of bands were quantified using Lumi-Imager and expressed as BLU (Biochemical light unit). Results were obtained from two individual experiments (n=4) and expressed as mean ± SEM.

* P<0.05;**P<0.01;***P<0.001 versus control.

#P<0.05 ; ##P<0.01###P<0.001 versus treatment group without antagonist addition.

5.3.6 Effects of G15 on regulation of intracellular cAMP level in UMR106 cells

We have shown that treatment of UMR 106 cells with E2 and the flavonoids failed to induce a rapid intracellular cAMP accumulation. In fact, treatment with E2 and naringin even led to a decreased level of intracellular cAMP ($P<0.05$, vs control) To investigate the role of GPR30 in regulating intracellular cAMP accumulation in UMR106 cells, the cells were treated with vehicle, PTH (10^{-8}M), 17β-estradiol (10^{-8}M) and Naringin (10^{-7}M), for 10 minutes with or without pre-incubation with G15 (10^{-6}M) for 20 minutes prior to the treatment.

Our findings showed that treatment of UMR 106 cells with PTH for 10 minutes induced a substantial increase in intracellular cAMP level (80-fold increase, $P<0.001$). Pre-incubation of UMR 106 cells with G15 not only failed to reduce this rapid induction of intracellular cAMP by PTH, but in fact led to a much higher degree of cAMP accumulation (140-fold increase, $P<0.001$). Treatment of UMR 106 cells with E2 for 10 minutes decreased the intracellular cAMP level to 0.72-fold ($P<0.05$ vs control), and pre-treatment with G15 abolished this opposite effect and restored the cAMP back to control level. Similar to E2, the inhibitory effect of naringin (0.77-fold vs control, $P<0.05$) in UMR 106 cells could be abolished by pre-incubation with G15.

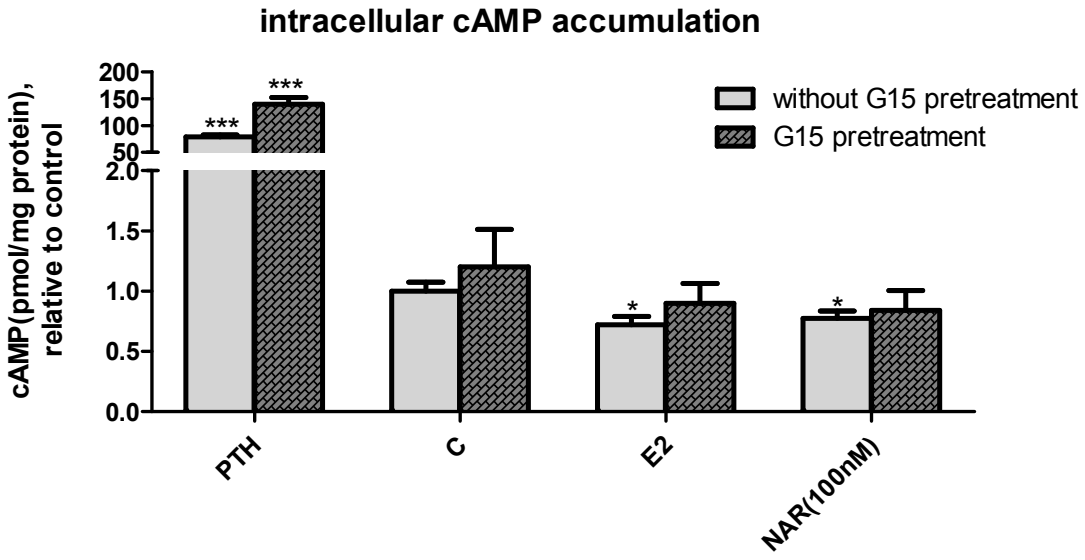


Figure 5.7: Effects of PTH, estrogen and naringin with or without G15 preincubation on intracellular cAMP production in UMR106 cells. UMR106 cells were treated with vehicle (C), Parathyroid hormone (PTH, 10^{-8}M), 17β-estradiol (E2, 10^{-8}M) or naringin (NAR, 10^{-7}M) for 10 minutes with or without preincubation with G15 (10^{-6}M) for 20 minutes prior to treatment. Cell lysates were collected at the end of experiment and intracellular cAMP accumulation were deteceted by ELISA kit. Relative cAMP concentration was shown as fold over vehicle control and normalized with the total protein content measured by Bradford assay. Results were obtained from 2 individual experiments (n=4) and expressed as mean $\pm$ SEM.

* $P<0.05$;***$P<0.001$ versus vehicle control.

5.4 Discussions

The current study evaluated the effects of GPR30 inactivation by GPR30 specific antagonist G15 in the actions of E2 and phytoestrogens in UMR106 cells. G15, along with the GPR30 agonist G1, was developed by Prossnitz and colleagues with the intention to investigate the functional roles and signaling mechanisms of specific GPR30 activation (Bologa et al. 2006; Blasko et al. 2009; Dennis et al. 2009). Both G1 and G15 displayed a high binding specificity to GPR30 with minimal binding to the classical ERα and ERβ, but only G1 has the ability to activate GPR30-mediated signaling in breast cancer cells. G15, in particular, was found to effectively inhibit the rapid cellular signaling induced by 17β-estradiol including calcium mobilization and PI3K activation in SKBr3 breast cancer cells which lack expression of ERα and ERβ but express GPR30 (Dennis et al. 2009). However, as mentioned, the functional roles of GPR30 in bone remained controversial, and few studies were conducted on the signaling pathway via GPR30 activation in osteoblasts. In this study, as we have found that the flavonoids could trigger rapid estrogenic signaling via a series of phosphorylation, we tried to investigate if these effects could be related to GPR30.

To understand the role of the GPR30-mediated signaling pathway in the genomic actions of estrogen via ERE-dependent transcriptional events, the transfected UMR106 cells was pre-incubated with G15 before treatment with the compounds. As naringin and icariin did not show induction on both ERα and ERβ-mediated ERE-luciferase activity on UMR106 cells at various concentrations tested, only genistein (10^{-6}M) were tested in this experiment. The results of pre-treatment with G15 suggested that GPR30 plays a role in the signaling pathways in UMR 106 cells exerted by estrogen as well as genistein that

lead to transcription of ERE-promoter containing genes. A clear relationship was found between GPR30 with ERα rather than ERβ in UMR 106 cells as G15 was able to partially block the stimulation of ERα-mediated ERE-dependent luciferase activity by our compounds but has no significant effects on the estrogen or genistein-induced ERβ-mediated ERE-dependent luciferase activity. In comparison, ICI administration was sufficient to completely abrogate the ERE-luciferase activity in UMR 106 cells as no further inhibition was found when the cells were pre-incubated with both ICI and G15. The results suggested that estrogen receptors played the main role in the induction of ERE-dependent transcription while GPR30 might act as one of the regulating factors in ERα-mediated signaling system.

It was not clear, however, whether GPR30 plays any role in mediating the ligand-independent actions of flavonoids in UMR 106 cells in activating ERα and nongenomic actions of estrogenic compounds on the initiation of rapid cellular signaling cascade. It was proposed that the genomic and nongenomic pathways mediated by estrogen via classical ER and GPR30 are connected to each other and regulate estrogen signaling in a coordinated fashion via rapid cellular signaling (Prossnitz et al. 2008). In light of the possible involvement of GPR30 in the signaling exerted by the flavonoids, we sought to find out the effects of G15 on the flavonoids-induced activation of ERα, MAPK signaling and PI3K/Akt signaling cascades.

To our surprise, our results showed that pre-treatment of UMR 106 cells with G15 not only failed to antagonize the rapid cellular signaling induced by the compounds, but even up-regulating each of these kinase-mediated responses. Treatment of UMR 106 cells with G15 alone were able to stimulate ERα phosphorylation at Ser118 by 1.8-fold,

ERK1/2 phosphorylation by 4.2-fold, and Akt phosphorylation by 2.1-fold. Combination treatment with the compounds and G15 seemed to potentiate the effects of the compounds, resulted in a more significant increase in these responses than treatment with E2 or the flavonoids alone.

G15 has been widely used in the studies of nongenomic estrogen signaling of GPR30 in breast cancer cells (Dennis et al. 2009), mesenchymal stem cells (Jenei-Lanzl et al. 2010) and other cell types (Gingerich et al. 2010; Peyton 2010; Lindsey et al. 2011). G15 was reported to reduce ERK phoshporylation that involved in mediating the rapid vascular effects stimulated by aldosterone (Gros et al. 2011). However, to our knowledge, this phenomenon of G15-induced increase in ERα, ERK1/2 or Akt phosphorylation has not been reported before in any of the studies on the effects of GPR30 and G15. These findings suggested that our initial speculation, i.e. flavonoids signal via GPR30 activation to induce rapid estrogenic downstream signaling, might not be the pathway exerted by the phytoestrogens in osteoblast cells. It is not known whether these effects were caused by the interaction of G15 with other factors or proteins apart from GPR30 to induce the rapid phosphorylation of the kinases. It is also possible that inhibition of GPR30 by G15 attenuated the activities of phosphatases or stimulated the activities of kinases, or the blocking of GPR30-mediated signaling was compensated by another estrogen related pathways which would activate the downstream signaling. The actual role of GPR30 as well as the actions of G15 in bone cells required additional research.

Intracellular level of cyclic AMP (cAMP) was found to be rapidly elevated in response to activation of GPR30 by E2 (Filardo et al. 2002; Thomas et al. 2005). However, the current knowledge of GPR30-cAMP-relationship and mechanisms were

based on experiments conducted in breast cancer cells, and studies elucidating the relationship between GPR30 activation and cAMP level in osteoblastic cell line were scarce. In fact, based on our study, neither estrogen nor the flavonoids could induce the accumulation of intracellular cAMP and the downstream DNA target CRE-mediated transcription in UMR106 osteoblasts. Only PTH was shown to vastly increase the cAMP level and CRE-luciferase activity, and the effects were of course attributed to PTH receptor which is also a G-protein coupled receptor itself. In contrast, estrogen and naringin was found to inhibit the basal cAMP level in UMR106 cells by around 30% and 23% respectively. This suggested that E2 and the flavonoids did not stimulate cAMP release in UMR 106 cells.

Our results showed that G15 pre-treatment led to a higher elevation of intracellular cAMP induced by PTH administration, while the decreased level of cAMP induced by E2 and naringin were restored to basal level by co-treatment with G15. The fact that G15 was able to modulate the effects of PTH, estrogen and naringin on cAMP level suggested GPR30 was involved in regulating the intracellular cAMP production in UMR106 cells. In contrast with the signaling event in breast cancer cells, the role of GPR30 in osteoblastic cells might be on the inhibition of the activity of adenylyl cyclase, leading to the decrease in cAMP production.

Together, we found that the ligand-independent activation of ERα and rapid cellular MAPK and PI3K/Akt signaling induced by the flavonoids did not require the activation of GPR30. However, GPR30 were found to be involved in modulating cell proliferation, differentiation and genomic ERE-dependent transcription, while regulating the rapid cellular signaling and intracellular cAMP level in a different manner from those of E2 signaling in UMR106 cells. The molecular mechanisms of GPR30-mediated signaling in osteoblastic cells required further investigation.

Chapter 6

Discussions and Conclusion

6.1 Discussions

Phytoestrogens are naturally occurring compounds found in plants, fruits or herbs that displayed a certain degree of estrogen-like properties either structurally or functionally. Among hundreds of them, the largest group of phytoestrogens, flavonoids, received much attention for their potential role as antioxidants and beneficial effects on cardiovascular diseases (Kris-Etherton et al. 2002), cancer prevention (Le Marchand 2002; Arts et al. 2005) and osteoporosis (Anderson et al. 1999; Songlin et al. 2009). Studies by our group have provided evidences that three members of flavonoids, namely naringin found in citrus fruits, icariin found in Herba Epimedii, and genistein found in soy products were potent in exerting osteoprotective effects in vivo and in vitro (Chen et al. 2006; Mok et al. 2010; Pang et al. 2010). However, their mechanisms of action in osteoblastic cells, particularly naringin and icariin, remained unclarified since they were found to have little effects on the classical genomic responses but somehow they were dependent to ER in exerting their anabolic effects on bone.

In the present study, we have confirmed the anabolic effects of these phytoestrogens on osteoblast growth, osteogenic differentiation and modulation of osteoclastogenesis. Using specific antagonists of ER and GPR30, we found that these estrogenic actions of these flavonoids on UMR106 cells required the involvement of the classical ERs as well as the membrane bound GPR30. The findings that ER and GPR30 involved led us to further investigate their mechanisms of action via estrogenic signaling by which these flavonoids exert their effects in bone cells.

6.1.1 Genomic ER signaling

To delineate their mechanisms of action via estrogenic signaling, we have first investigated the possible signaling of phytoestrogens via ER-mediated genomic pathway. The competitive assay suggested that only genistein bound both ERα and ERβ, while naringin and icariin did not bind any of the two. Studies on the structure/activity analyses of various estrogenic flavonoids have shown that the existence of the hydroxyl groups, in particular at the positions of 7 and 4' of the flavan or isoflavan backbone, appears to be crucial for the estrogenic property of that compound as they correspond to the hydroxyl groups at position 3 and 17 of estradiol (Miksicek 1995; Zand et al. 2000; Ming et al. 2013). Any modification on these hydroxyl groups such as methylation and conjugation would lead to a diminished or even lost in estrogenic activity. Among the flavonoids in our study, only genistein has unaltered hydroxyl groups at the 7 and 4' positions. The structure of naringin contains a rutinose group conjugated to hydroxyl group at position 7, while both the hydroxyl groups of icariin at position 7 and 4' were substituted by glucosyl group and methoxyl group respectively **(Figure 6.1)**. Thus, it was not surprising that the bulky glycosides of naringin and icariin hindered their interaction with LBD of ERs, while the simple structure of genistein enabled binding to the receptors with certain affinities.

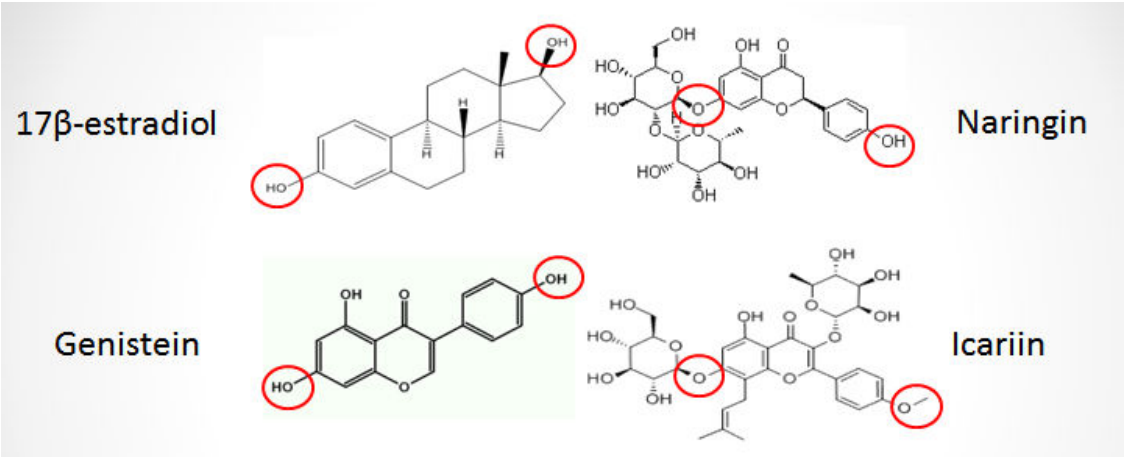


Figure 6.1: Structure of the flavonoids with the side groups at position 7 and 4' were circled in red. Also circled were the hydroxyl groups at position 3 and 17 of estradiol.

Nonetheless, the binding results alone could not rule out the possible involvement of naringin and icariin in the genomic pathway as studies have shown that even without direct interaction with nuclear ERs, some estrogenic compounds such as Ginsenoside Rg1 were still able to induce ERE-mediated transcription (Lau et al. 2008). Thus, we have conducted a transient transfection of ERE-luciferase vector along with ERα or ERβ plasmids into UMR106 cells in order to investigate the abilities of flavonoids to trigger ERE-dependent transcriptional activities. As UMR106 cells showed a low endogenous level of estrogen receptors (Davis et al. 1994), it was convenient to manipulate over-expression of each estrogen receptor subtype with low background. The results of luciferase activity were found to be consistent with the ER binding assays. Genistein were able to induce both ERα and ERβ-mediated ERE-luciferase activity, while naringin and icariin at most concentrations demonstrated little effects on ERE-luciferase activity via both ERs. Furthermore, our immunoblotting results showed that naringin, icariin and genistein could induce the phosphorylation of ERα at Ser118, a site which could be

phosphorylation in response to ligand-independent activation of ERα. Taken together, these results suggested that the actions of naringin and icariin were mediated through the ligand-independent activation of ERα without the involvement of classical ER pathway, while the actions of genistein involved both.

6.1.2 Rapid non-genomic signaling

We next focused on the abilities of the flavonoids to signal via non-genomic arm of ER signaling. As introduced, the communication between ER and membrane-coupled receptor tyrosine kinases (EGFR, IGF-1R) enables the activated ER to signal via MAPK, PI3K and other rapid cellular cascades (Hall et al. 2001). Essentially, the ligand-independent phosphorylation of ERα at Serine 118 requires the activation of MAPK signaling as well.

In this study, UMR106 cells treated with the flavonoids as well as 17β-estradiol activated the MAPK signaling via rapid phosphorylation of ERK1/2 within 5 minutes incubation and the signals sustained for at least 1 hour. In the bone, activation of the MAPK pathway by estradiol are associated with anti-apoptotic effects on osteoblasts and osteocytes (Kousteni et al. 2001) but pro-apoptosis effects on osteoclasts (Chen et al. 2005). In addition, MAPK activation has been reported to positively regulate the in vitro differentiation of osteoblasts and in vivo bone formation via stimulation of Runx2, an essential bone formation marker (Ge et al. 2007). Kousteni et al reported that treatment with E2 induced phosphorylation of ERKs in osteocytes and osteoblasts that linked to effective prevention of cell apoptosis. This anti-apoptotic effect could be abolished by ER inhibitor ICI182780 as well as ERK phosphorylation inhibitor PD98059 and Src inhibitor PP1, indicating the importance of ERK activation in mediating ER-dependent estrogenic

effects on osteoblasts. Another important and unexpected result from the study is that the biological effects of ERK activation could be dissociated from the transcriptional activity of ERα, i.e. independent of ERE-mediated transcription (Kousteni et al. 2001). In association with our current study, it provided a support that the estrogenic responses of flavonoids in UMR106 cells might be mediated at least in part via non-genomic MAPK signaling rather than the direct ERE-mediated genomic pathway. In addition, the abilities of icariin to induce ERK phosphorylation and the subsequent ligand-independent ER activation in bone cells were also supported by other recent studies (Feng et al. 2013; Song et al. 2013) However, further study is required to elucidate the precise downstream target transcriptional factors activated by MAPK signaling and the association with biological responses in osteoblasts

In contrast with the anti-apoptotic effects of MAPK signaling, PI3K/Akt pathway was associated with pro-survival signals and osteogenic differentiation (Dufour et al. 2007). In the current study, UMR106 cells treated with 17β-estradiol and genistein showed reduced level of Akt phosphorylation, while naringin and icariin elevated the phosphorylation of Akt on the initial 30 minutes incubation. To address this effects of E2 in UMR106 cells that seemed to unparallel with other cell lines, Sunters suggested that in UMR106 cells where ERs were in short supply, E2 might compete for ERα with IGF1-R, leading to the depletion of ERα to sufficiently complexed with IGF1-R in response to extracellular signals, thereby inhibiting the downstream signaling induced by ERα/IGF1-R complex including PI3K/Akt pathway (Sunters et al. 2010). Thus, it was possible that in our study, E2 and genistein bound with the limited number of ERα to enhance its nuclear translocation and transcriptional events, whereas naringin and icariin served as

extracellular signals to induce the nongenomic estrogenic responses and will not influence the ERα availability in cytoplasm to form the initiation complex of PI3K/Akt signaling. The consequences of this difference in PI3K/Akt signaling between E2 and genistein with the flavonoid glycosides might provide an alternate mechanism for these glycosides to exert their osteogenic effects in UMR106 cells.

6.1.3 Involvement of GPR30

As we found that the flavonoids, particularly naringin and icariin, did not seem to signal via ER-mediated classical genomic pathway but rather trigger the non-genomic rapid cellular signaling cascades, we next tried to relate the non-genomic rapid signaling with the novel G protein estrogen receptor GPR30. Studies have found that E2 bind GPR30 and proposed that GPR30 might mediate the non-genomic signaling mechanisms induced by E2 (Thomas et al. 2005; Prossnitz et al. 2008), leading to the speculation that these two pathways might converge and that the flavonoids might selectively activate GPR30. Filardo et al showed that E2 activated ERK phoshporylation in SKBr3 cells which lack expression of classical ERs but express GPR30, suggested that GPR30 mediated the estrogenic signaling at least in part in breast cancer cells (Filardo et al. 2000).

The results on cell proliferation and differentiation suggested that GPR30 do play a role in the osteogenic effects of the flavonoids in UMR106 cells. However, pre-treatment with G15 yielded unexpected results on the phosphorylation of ERα at Ser118, MAPK signaling and PI3K/Akt signaling regulated by the flavonoids. The results showed that G15 not only failed to attenuate the phosphorylation events, but led to overall increase in the phosphorylation of these proteins and kinases, suggested that GPR30 regulated the

rapid signaling events differently with the non-genomic E2 signaling in UMR106 osteoblasts.

In fact, controversies regarding the role of GPR30 as an estrogen receptor and their downstream signaling in response to E2 treatment have never been resolved. For instance, using U2OS, CHO and COS-7 cells transfected with GFP-tagged GPR30 as well as MBA-MD231 and Hec50 cells that endogeneously express GPR30, Otto et al showed that GPR30 did not bind 17β-estradiol and did not trigger non-genomic signal including cAMP accumulation, calcium elevation, and ERK activation in response to E2 administration in these cells, suggesting that GPR30 are not the target of E2 (Otto et al. 2009). These negative in vitro results were supported by another study (Pedram et al. 2006). Pedro and colleagues found that endothelial cells isolated from ERα/ERβ knockout mice, which still express GPR30, failed to bind E2 and induced the cAMP, MAPK and PI3K/Akt signaling in response to E2. Furthermore, they found that MCF7 cells transfected with siRNA of GPR30 did not prevent the rapid cellular signaling induced by E2. These controversies were addressed and extensively discussed in the review of Langer on the current researches on GPR30 (Langer et al. 2010). These studies were in contradiction with the positive results of others as discussed previously. Nonetheless, our current results on UMR106 cells were in consistent with the findings that GPR30 is not required for the non-genomic rapid estrogenic responses induced by the phytoestrogens. Whether there is a tissue or cell-specificity on the GPR30 role in estrogenic signaling remained unknown. Moreover, discrepancies in the in vivo results of GPR30-knockout mice in the bone development between several groups (Mårtensson et al. 2009; Windahl et al. 2009; Ford et al. 2011) suggested that the exact roles of GPR30

in bone homeostasis still need to be further clarified in the research field.

In this study, we found that while GPR30 displayed certain involvement in mediating the biological responses induced by the flavonoids in osteoblasts, it was not responsible for the non-genomic signaling induced by the compounds but rather enhanced their rapid activation of MAPK and PI3K when it was inactivated. Further study would be required to elucidate the functional roles and the signaling mechanisms mediated by GPR30 in osteoblasts.

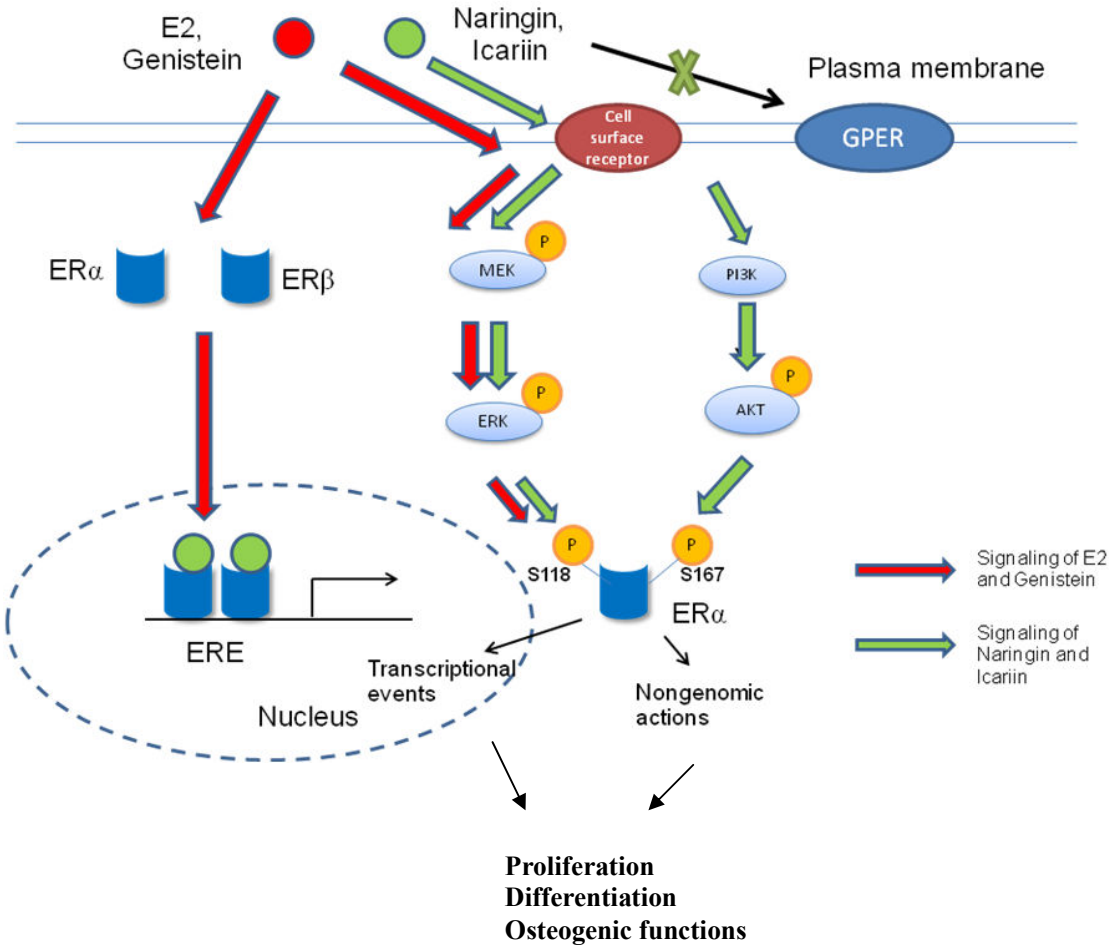


Figure 6.2: Proposed mechanisms of action of naringin, icariin and genistein in rat osteoblastic UMR106 cells

6.2 Conclusions

In conclusion, we have demonstrated that the phytoestrogens naringin, icariin and genistein exerted positive effects on osteoblastic cell proliferation, osteogenic differentiation and modulation of osteoclastogenesis. The study also demonstrated that while the actions of genistein were more similar to 17β-estradiol in promoting both ER mediated genomic signaling and non-genomic rapid cellular signaling via MAPK pathway, naringin and icariin preferentially activating non-genomic estrogenic signaling pathways including MAPK signaling and PI3K/Akt pathway with little effects on classical genomic pathway. The actions of phytoestrogens required the classical estrogen receptors and they were able to activate ERα in a ligand-independent manner. While GPR30 was involved in mediating the effects of the phytoestrogens, it was not required for the rapid cellular signaling induced by the flavonoids and was found to regulate the activation of these kinase-mediated pathways in a different fashion and mechanisms with the non-genomic estrogenic signaling **(Figure 6.2)**.

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