CHAPTER II

LITERATURE REVIEWS

A. *Pueraria mirifica*

1. Plant description and biological effects

Pueraria mirifica is a type of hard vine that grows up and climbs around large trees. Palmate type leaves, with three leaves in one petiole; its leaves are simple (ovate) and angle leaf tips. Flowers are bluish purple, a cluster of 30 centimeters length at the end of its twig. The flower is similar to a bean flower, each composes of five petals, the outer part is the biggest one, the two petals on both sides are curved, and the interior petals wrap an ovary. The plant flowers from February to March and produces pods in April. Aged brown flat pods produce 3-5 seeds. Tuberous roots are in different sizes. Its appearance is similar to a yam root. Inside, the root is white and the taste can cause dizziness. Root sizes are various depending on the soil condition or the environment and the time of cropping. A single tuberous root can weigh as much as 100 kg. The shape and size of PM is diverse and often differs within each environment (anonymous, 2006).

PM is a Thai herb that has long been popularly consumed among Thai people. Its scientific name is *Pueraria mirifica* Airy Shaw et. Suvatabandhu (synonym: *Pueraria candollei* Wall. ex Benth var *mirifica* (Airy Shaw & Suvat.) Niyomdham). It has been used in menopausal women for estrogen replacement therapy. The active ingredients were found in these plant tuberous roots. It has been shown in animal experiments and clinical trials that the crude ethanol plant extract has estrogenic activity (Muangman and Cherdshewasart, 2001; Malaivijitnond et al., 2004). A study has demonstrated that miroestrol was the first active ingredient; it has a strong estrogenic effect when used in immature female mice and ovariectomized rats. These effects were found to be similar to the clinical trial reported in menopausal women (reviewed by Cherdshewasart et al., 2004). In 2000b, Chansakaow and coworkers found that there is another derivative of miroestrol, deoxymiroestrol (Figure 2-1), which has stronger estrogenic activity than miroestrol. There are many researches conducting on active ingredients of this plant; they found that daidzin, daidzein, genistein, genistin, coumestrol, puerarin, mirifin, kwakhurin,

mirificoumestan are the most active constituents in PM (Tahara et al., 1987). Nowadays, there are number of studies reported on the effects of PM on the reproductive organs, they found that PM can stimulate the proliferation of vaginal and uterus epithelium in female rats (Malaivijitnond,et al., 2004). In addition, they found that PM can induce vaginal cornification in ovariectomized rats (Malaivijitnond et al., 2006). In 1986, Smitasiri and coworkers found that PM can inhibit follicle growth and ovulation in female rats. Later on, Trisomboon et al. (2004) found that feeding on PM can prolong menstrual cycle in adult cyclic cynomolgus monkeys. There is a report claimed that PM can reduce menopausal symptom in women such as hot flush, sleep disorder and skin dryness (Muangman and Cherdshewasart, 2001). In 2004, Malaivijitnond and coworkers confirmed that PM affected accessory sex organs and gonadotropin levels in both sexes of rats similar to estrogen. They also found that PM possessed estrogenic activity on both uterotrophic and vaginal cornification assays in female rats (Malaivijitnond et al., 2006). Moreover, it has been demonstrated that PM has estrogenic action on sexual skin coloration in menopausal cynomolgus monkeys (Trisomboon et al., 2006).

Furthermore, from the study in cancer cells, it has been found that PM has dual effects on the growth of MCF-7, human breast cancer cells; at low dose, it can stimulate cell proliferation but at high dose it reduces cell proliferation (Cherdshewasart et al., 2004). In 2005, Jeon and coworkers studied the antitumor effect of PM; they believed that this effect was from spinasterol, an active component in theirs PM. Additionally, the effect of PM on the requirement of metabolic activation for estrogenic activity has been done. They found that PM can induce estrogenic activity in MCF-7 human breast cancer cells and HepG2 human hepatoma cells, but it can not induce estrogenic activity in recombinant yeast cells (Lee et al., 2002).

2. Chemical compositions of *Pueraria mirifica* herb

The chemical compositions of PM are flavone group such as daidzein, genistein, coumestrol, daidzin, genistin, puerarin, mirifin, kwakhurin, mirificoumestan and the dominant compounds, miroestrol and deoxymiroestrol (Figure 2-2). The latter two compounds have been believed to contain estrogenic effects greater than other phytoestrogens.

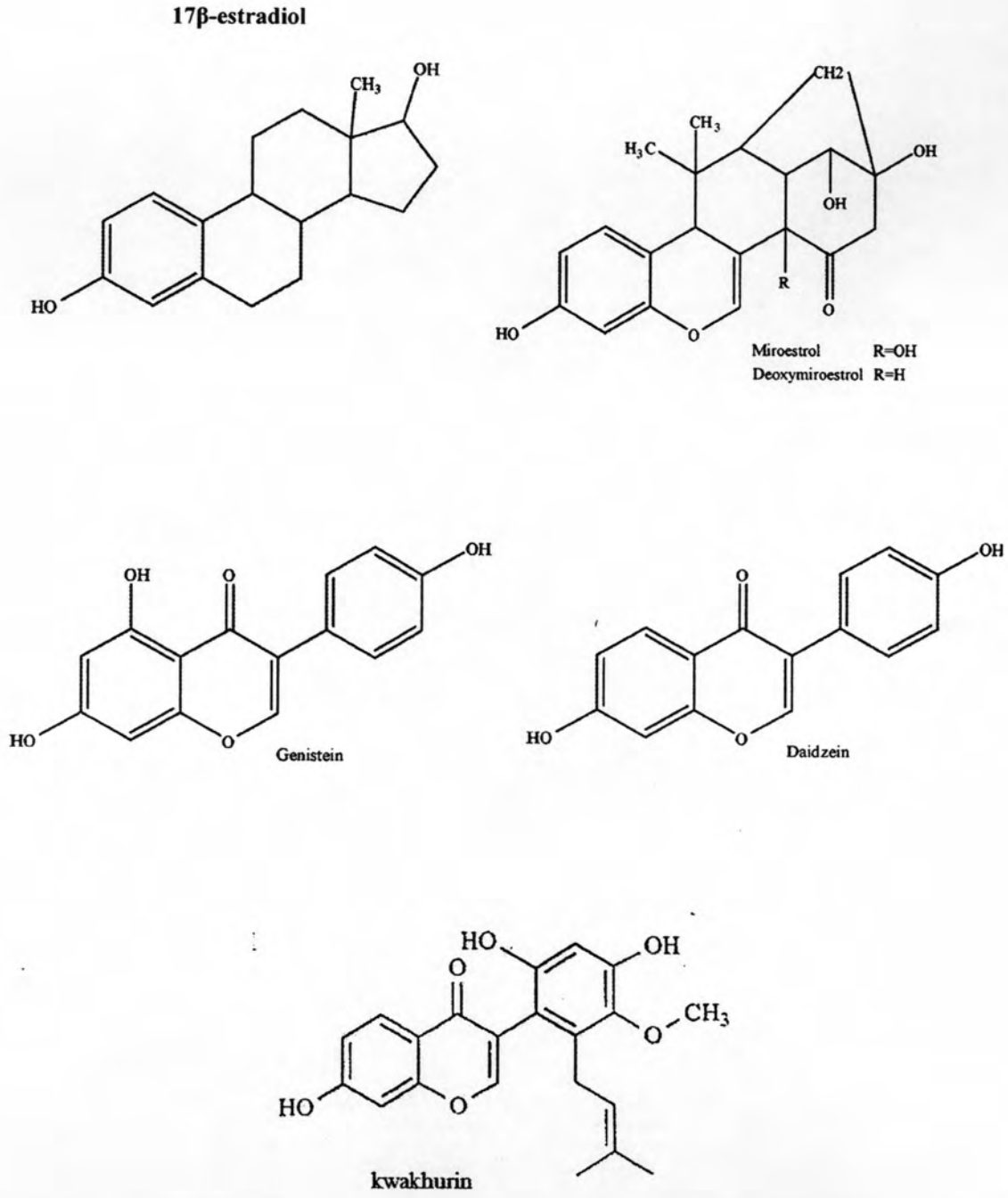

Figure 2-1 Comparison of the chemical structures of 17β-estradiol, miroestrol and deoxymiroestrol, genistein, daidzein and kwakhurin.

B. Phytoestrogens

Phytoestrogens are plant substances that can mimic action of estrogen and able to induce biological responses in mammal. Theirs structural are diphenolic compounds similar to endogenous hormone. The phytoestrogen can bind to estrogen receptor; and it has both estrogenic and anti-estrogenic effects, in that it can stimulate the uterus growth while reduce the breast cancer cell growth (reviewed by Terreaux et al., 2003). There are reports evaluated the effects of phytoestrogens in inducing transcriptional effects. They found that phytoestrogens have a lower ER-α affinity compare to estradiol (Kuiper et al., 1998). They found that phytoestrogens, especially isoflavone, have a higher affinity to ER-β; the ranking of phytoestrogen upon ER-β binding is: estradiol >> zearalenone = coumestrol > genistein > daidzein >apigenin > kaemferol > formononetin = ipriflavone = quercetin; for ER-α binding: estradiol >> genistein = coumestrol > zearalenone > daidzein > apigenin = kaemfol > formononetin = ipriflavone = quercetin (reviewed by Kuiper et al., 1998).

The studies on the effects of phytoestrogens on the estrogenic and anti-estrogenic actions are still controversial. However, it has been believed that the phytoestrogen acts as an antagonist to ER-α and more agonist to ER-β. With consideration from many studies, the protective effect of isoflavones against breast and prostate cancers may be depended on the differential tissues distribution of both ER subtypes (Enmark et al., 1997; Kuiper et al., 1996; Morito et al., 2001). The phytoestrogens can be classified into different groups as shown in Figure 2-2; however, only those found in PM were reviewed herein (i.e. kwakhurin, miroestrol, deoxymiroestrol, daidzein and genistein).

1. Kwakhurin

Kwakhurin is the chemical compound found in PM, its structure is similar to 17β-estradiol (Figure 2-1). It had proliferative effect on the growth of MCF-7, human breast cancer cells (Chansakaow et al., 2000a). It is the main compound of PM that contained rejuvenating property in Thai medicine.

2. Miroestrol

Miroestrol was first isolated from tuberous roots of PM. In animal studies, miroestrol has been shown to contain 25 times more potent than that of 17β-

estradiol tested in rat vaginal cornification assay, approximately 3 times than that of stilbesterol in the immature mouse uterine growth test, and 2/3 that of stilbesterol in the rat vaginal cornification test (reviewed by Miers, 2004). In promoting mammary duct growth in the rat, its activity was about 70% of the 17β-estradiol's activity when administered by subcutaneous injection; and was 2.2 times as active as estrone when test in the mouse (reviewed by Miers, 2004). Pure miroestrol has been reviewed to be first synthesized from PM and the molecular formula was determined to be $C_{20}H_{22}O_6$ with a melting point of 268 degrees. However, miroestrol was not claimed as a steroid and its biological activity is probably a consequence of accidental features of molecular geometry (reviewed by Miers, 2004). In which, Osborne and Hallaway (1962) has reported that miroestrol has no effect on protein synthesis in plant cells comparable with that reported from animal cells.

3. Deoxymiroestrol

Deoxymiroestrol is a dominant active compound in PM which is the metabolic precursor extracted from miroestrol. Researchers believed that during extraction process of miroestrol, the precursor deoxymiroestrol could be easily through facile aerial oxidation and then become miroestrol (Chansakaow et al., 2000b). In 2005, Mutsumura and coworkers investigated the estrogenic property of eight phytoestrogens in MCF-7 human breast cancer cells; they found that deoxymiroestrol contained estrogenic activity higher than that of 17β-estradiol and miroestrol in every assay.

4. Genistein

Genistein is an isoflavone, the one component in PM found more abundant in soy products, it has higher affinity binding to ER-β than ER-α (reviewed by McCarty, 2006). Genistein acts as estrogen agonist both *in vivo* and *in vitro*; for instance, it had proliferative effect on culture MCF-7 human breast cancer cells (Hsieh et al., 1998). There is a report that genistein can inhibit MDA-MB-231 breast cancer cells growth *in vitro*, but the same concentration can not inhibit the growth of same cells *in vivo* (Santell et al., 2000). In 2001, Diel and coworkers have found that genistein can increase the uterine weight and stimulate uterine estrogen-dependent gene expression. They concluded that this isoflavone acted as a weak ER agonist in

ovariectomized rats. The effect of genistein on the proliferation of normal human endometrial cells and Ishikawa cells, human endometrial cells has been done, they found that genistein increase the endometrial cell proliferation at high concentrations (10^{-8} to 10^{-6} M) the results similar to the *in vivo* study (Kayisli et al., 2002). Power and Thompson (2003) study in MCF-7 and T47D cell, human breast cancer cells, they found that genistein increased proliferation at low dose (0.05-50 μM), but decreased cells proliferation at high dose (100 μM) in both two cells types. In 2005, Edmunds and coworkers found that genistein (1 nM to 1 mM) can stimulate aromatase activity in human endometrial stromal cells.

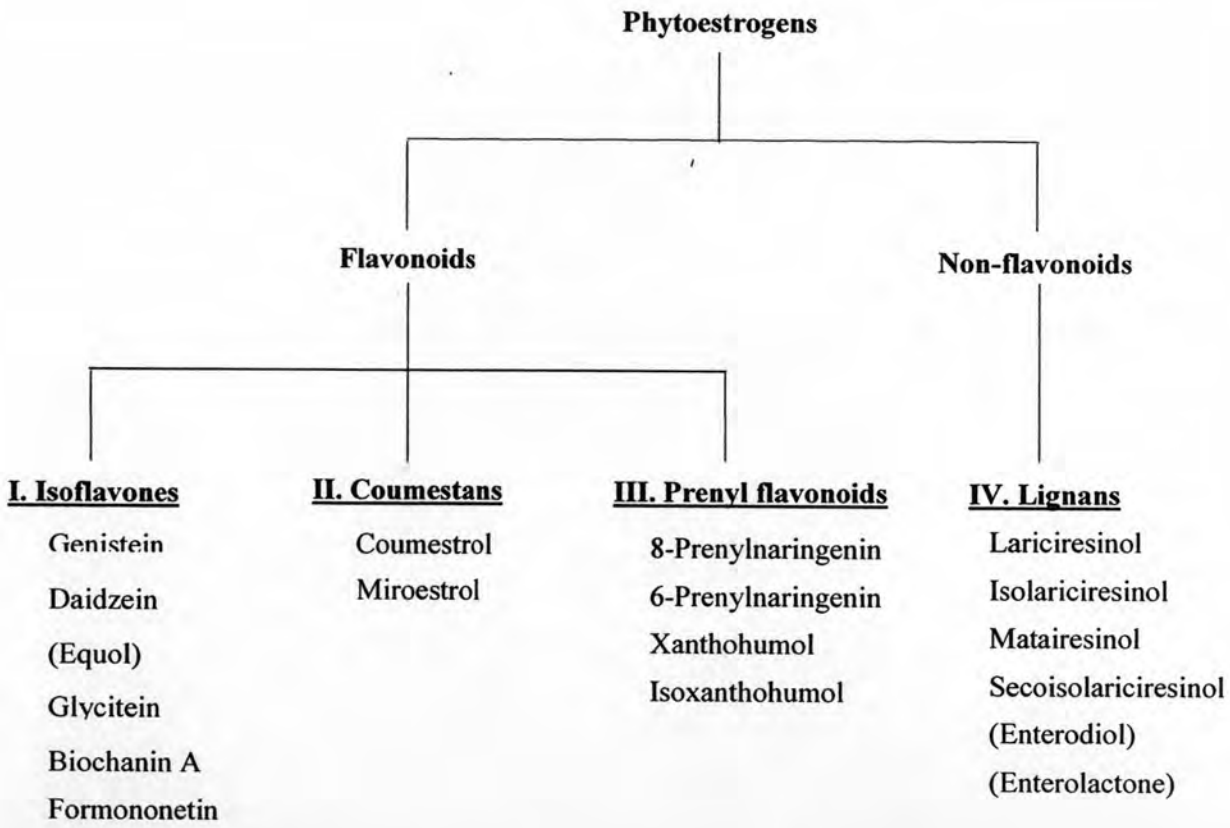

Figure 2-2 Classification of various groups of phytoestrogens and theirs members (modified from Terreaux et al., 2003)

5. Daidzein

Daidzein is an isoflavone, like genistein, has been described as a weak phytoestrogen and binds to ER-β with higher affinity than ER-α (reviewed by Lamartiniere et al., 2002b). Daidzein is lacking of hydroxyl group, this resulted in decreased affinity to bind receptor (Terreaux et al., 2003). The metabolite of daidzein, namely equol can bind to ER-α comparably to genistein. The equol was found to act as a strong estrogenic and genotoxic to Ishikawa cells (Lehmann et al., 2005). Daidzein occurs mainly in soy as their respective glycosides, it has negative effect on binding to both receptor subtypes (Morito et al., 2001). In 2002a, Lamartiniere and colleagues concluded that the supraphysiological concentration of daidzein has no toxic effect on the female reproductive tract and it can provide a protective effect against chemically induced mammary cancer in rats.

C. Estrogen

1. Estrogen effects and its synthesis

Estrogens are steroid hormones that exert a wide range of effects throughout the body, especially on reproductive system. Estrogens are required for normal female sexual maturation; they promote growth and differentiation of the breast, uterus, fallopian tubes, vagina, and ovaries (Carr, 1998). Moreover, estrogens are important for bone maintenance (Turner et al., 1994) and have a protective role in the cardiovascular system (Farhat et al., 1996). In non-pregnant premenopausal women, estrogens are primarily synthesized in the ovaries, using cholesterol as a precursor (Figure 2-3). The most potent and dominating estrogen in humans is 17β-estradiol, but also lower levels of the estrogens, estrone and estriol. Following synthesis in the ovaries, most estradiol are bound to plasma proteins and transported to target tissues. The majority of the estradiol (60%) is bound to serum albumin, 38% is bound to sex hormone-binding globulin, and 2-3% is free (Carr, 1998). Steroid hormones are lipophilic and have a low molecular weight that enabled them to diffuse freely through the cell membrane without the requirement of specialized transport systems. In both men and women, estrogens are also synthesized locally in non-endocrine tissues such as the brain, adipose tissue and liver by the conversion of androgens to estrogens due to the presence of the aromatase cytochrome P450 enzyme (Figure 2-3).

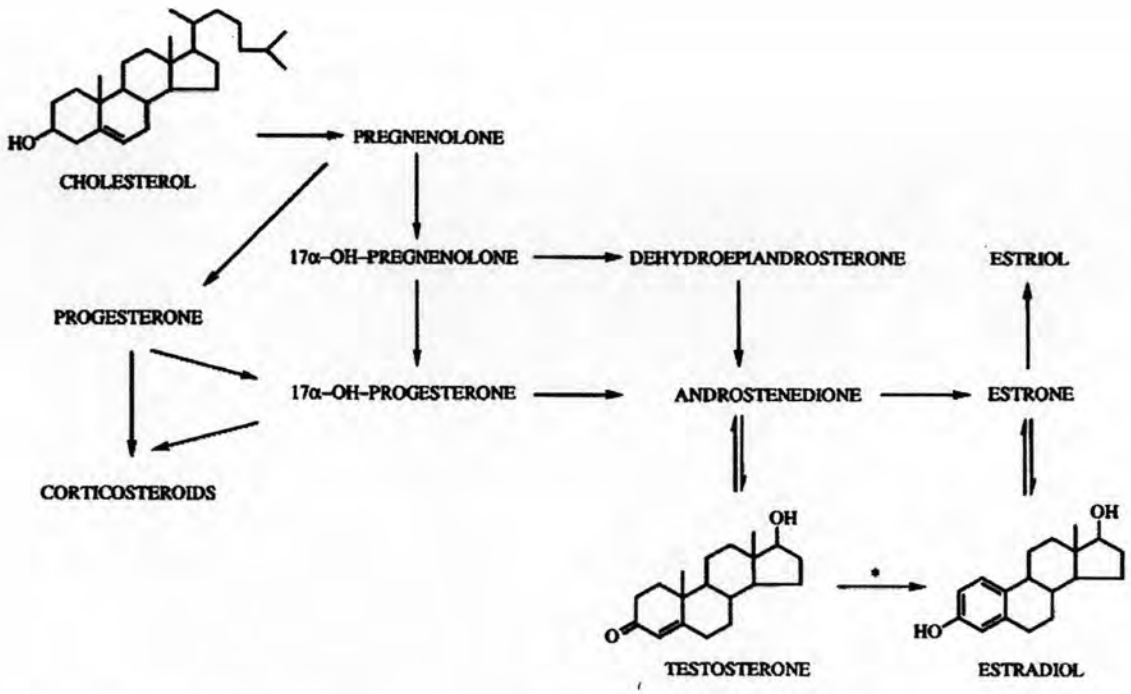

Figure 2-3 The biosynthetic pathway of the estrogens. Cholesterol is the main precursor and the enzyme aromatase cytochrome P450 (*) is responsible for the conversion of testosterone to estradiol.

2. Estrogen receptor

The presence of estrogen receptors (ERs) was first suggested by the work of Jensen and colleagues (reviewed by MacGregor and Jordan, 1998). In the mid-1980s, the first ER cDNA was cloned (Walter et al., 1985) and this receptor, presently known as ER-α. It was not until recently that a new ER subtype, ER-β, was discovered and cloned (Kuiper et al., 1996). The ERs belong to a large family of transcription factors, the nuclear receptor superfamily, that are intracellular and share many common properties such as nuclear localization and sequence specific DNA binding (Druege et al., 1986). The nuclear receptor proteins are composed of multiple functional domains and are characterized by the highly conserved DNA binding domain, which targets the receptor to specific DNA sequences known as hormone responsive elements (Druege et al., 1986).

3. Mechanism of action

Basically, estrogen mediates its activities *via* binding to a specific nuclear receptor protein, the estrogen receptor, which is encoded by two genes (ER-α and ER-β) that function as transcription factors to regulate the expression of target genes (Osborne et al., 2001a). On ligand binding, ER undergoes conformational changes and dissociates from the inactive ER-hsp90 complex. The activated ER enters the nucleus as a homodimer or heterodimer, then binds to a specific DNA sequence, the estrogen response element (ERE), and stimulates estrogen-target gene expression. The two ERs appear to have unique tissue distributions and their own sets of specific functions. Knowledge of these functions and different structures of phytoestrogen (Fig.2-1) might aid in the development of receptor-specific selective estrogen receptor modulators (SERMs) (Barkhem et al., 1998; Kuiper and Gustafsson, 1997; Nilsson et al., 1998). The two estrogen receptors, ER-α and ER-β, have similar overall structures (Figure 2-4), displaying a high degree of amino acid conservation in the central DNA-binding domain (DBD) and moderate conservation in the ligand-binding domain (LBD; C-terminus), but considerable divergence in the amino-terminus. Not surprising, therefore, ER-α and ER-β interact with the same DNA response elements and exhibit similar, but not identical, ligand binding characteristics. Although a specific physiological role for ER-β remains to be defined, its identification has provided a potential explanation for the biological actions of estrogen(s) in cells where no immunoreactive ER-α could be detected. Interestingly, preliminary localization studies have revealed that there are many tissues in which both ER subtypes are co expressed. Thus, the impact of ER-β on estrogen biology is likely to occur as a consequence of 1) direct actions of ER-β, where it is responsible for regulating target gene transcription; and 2) indirect activities, where ER-β modulates ER-α action in tissues where they are coexpressed (Reviewed by Hall and McDonnell, 1999). The mechanisms of action of estrogen can be classified into genomic and non-genomic.

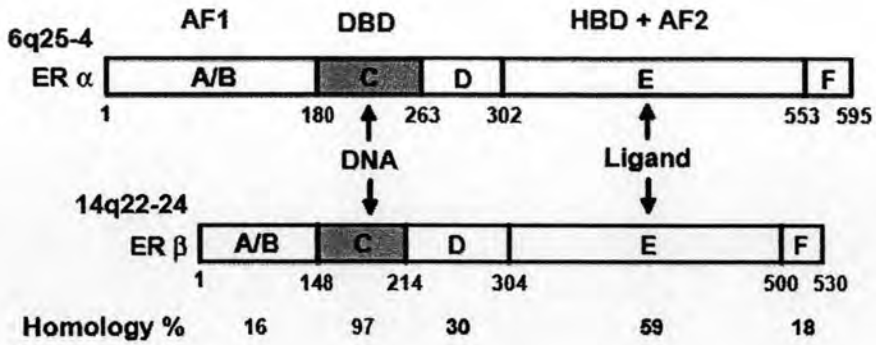

Figure 2-4 Structures and functions of estrogen receptors. The human estrogen receptors ER-α and ER-β contain five functional domains (A–E) as other members of the nuclear receptor superfamily and an additional domain F in their C-terminal part. The binding of estrogen in the hormone-binding domain (HBD) induces a trans-conformational change of the whole molecule allowing unmasking of the activating function 1 (AF1) in domain A/B by removal of chaperone (HSP90), dimerization, activation of activating function 2 (AF2) in the C-terminal part of the E domain and binding to estrogen-responsive element (ERE) on DNA via domain C. (Platet et al., 2004)

3.1 Genomic

The genomic actions of estrogen are mediated via intracellular ER-α and ER-β receptors: following a conformational change, receptor-estrogen dimers bind to a palindromic DNA sequence which, together with several others co-factors, leads to alterations in the gene transcription of estrogen-responsive elements (Driggers and Segars, 2002). These genomic actions, which are exerted over a time-scale of hours or more, are responsible for the actions of estrogen.

3.2 Non-genomic

It was found that the non-genomic action of estrogen involved alterations in the activity of G-protein-coupled receptor transduction cascades, diverse protein kinases, adenylyl cyclase and nitric oxide (Driggers and Segars, 2002). They were also found their modulation of current flux through ion channels, and interactions with neurotrophins (Nadal et al., 2000). These actions which can evoke

further downstream changes in cellular gene expression, at least partially, are mediated by membrane-localized receptors closely-related to intracellular ER-α and ER-β sites, though other mechanisms probably also intervene (Nadal et al., 2000; Wise et al., 2001). As an additional complication, it should be mentioned that ER receptors are susceptible to activation by phosphorylation, providing a potential mechanism for their estrogen-independent recruitment by a variety of intracellular signals (Segars and Driggers, 2002).

4. Effect of E_2 on reproductive system

Estrogens play important roles in the reproductive organ, especially on endometrial cells. The effects of E_2 on the target organs includs stimulating cell growth, differentiation, and control general homeostasis of reproductive and other systems. In the uterus, E_2 had effects in producing cyclical wave of cell proliferation and differentiation in preparation for implantation (O'Brien et al., 2006). Genes that are modified by estrogen are likely to be important players in the monthly cyclic development of the endometrium and possible leading to the pathogenesis of endometrial disorders (Vadlamudi et al., 2004). The action of E_2 is through both ER subtypes, which effects of E_2 may be depend on change of ERs (Hall and McDonnell, 1999).

D. Endometrial cell

1. Normal endometrial cell

The endometrium is comprised of zones that include the functionalis (shed on a monthly basis) and the basalis (believed to be the origin of cells for regeneration of the tissue) (reviewed by Giudice, 2006). The endometrium is composed of both glandular epithelial and stromal cells, which in this study we used a part of glandular epithelium. Both glandular epithelial and stromal cells demonstrated marked expression of ER-α mRNA. ER-β mRNA expression in glandular epithelial cells was weak compared with that of ER-α (Matzusaki et al., 1999). During the proliferative phase, endometrium is stimulated by high levels of circulating E_2, and then after ovulation in the early secretory phase, it is the target of low, but rising, of circulating levels of progesterone (P) and E_2. Thus, genes regulated in early secretory

and proliferative endometrium phases may be regulated by E_2 and/or P. Steroid hormones are the systemic factors that drive the endometrium through the characteristic sequential phases of the menstrual cycle (reviewed by Critchley et al., 2006). Steroids interact with their target cells via specific nuclear receptors with consequential initiation of gene transcription and a cascade of downstream molecular and cellular events. Estrogen receptor (ER) and progesterone receptor (PR) expression is under dual control of estradiol and progesterone, and along with the androgen receptor (AR) varies both temporally and spatially across the menstrual cycle (Critchley et al., 2001). Although a functional role for endometrial glands has been established in most mammals, mechanisms regulating their development in domestic animals, laboratory animals, and humans are not well understood (reviewed by Spencer and Bazer, 2004).

Guseva et al. (2003) stated that the organ physiology of swine is somewhat similar to that of humans; it has thus become the favored model for dermatologists, gastroenterologists, and obstetricians. In addition, porcine epithelial cell physiology and the mean length of its estrous cycle are similar to those in humans (Guseva et al., 2003), it has been used widely as model systems for *in vitro* analysis of pregnancy paradigms, actions of hormones, prostaglandin secretion, and epithelial cell-stromal cell interactions (Bowen et al., 1996; Reed et al., 1996; Zhang et al., 1991). Moreover, Tarleton et al. (1998) determined the effect of ovariectomy on the uterine growth of neonatal pig, and they concluded that at birth until post-natal day 120, the hormone had no effect on genesis of uterine glands or related endometrial morphogenetic events. During this time, the transformation of the porcine uterine wall from structural infancy to histoarchitectural maturity was also occurred (reviewed by Spencer et al., 1992). So in this study, endometrial glands were collected from pig aged about 3 months to minimize the cyclical change and the endogenous hormone effects. Another benefit of using porcine epithelial gland culture was that the pig uteri were easily to obtain (i.e. from slaughter house) and it could provide a high conformity of cells for study.

2. Endometrial cancer cell

Endometrial cancer is the most common malignancy in the female genital tract. The development of this disease involved with exposure to unopposed

estrogens and progesterone have been utilized in hormone-dependent cancer (reviewed by Castro-Rivera and Safe, 1998). As stated previously, the endometrium is a hormone-dependent tissue and most of the endometrial cancers are hormone-dependent tumors. These tumors express ER and/or progesterone receptor, and these receptors are the main target for the development of novel substances for treating such tumors. Among available models, endometrial cancer cell has long been recognized i.e. Ishikawa cell (reviewed by Vollmer, 2003).

The human endometrial carcinoma cell line, RL-95, is an estrogen receptor (ER)-positive adenocarcinoma derived from the primary tumor of a 65-year-old Caucasian woman (reviewed by Engel et al., 2005). These cells can be used as a model for investigating the effects of treatment on the cell proliferation and invasive properties of endometrial cancer (Park et al., 2000). These cell lines exhibit ER-α and ER-β similarly to Ishikawa cells, which were used to investigate the effects of 4-hydroxytamoxifen, raloxifene (SERM) on the cell proliferation (Leblanc et al., 2007). However, the effects of phytoestrogens on the RL-95, endometrial cancer cells are not fully understood.