กลไกของฮอร์โมนเพศชนิดสเตียรอยด์ในการควบคุมการแสดงออกของยีนผ่านทางไลน์-1

นางสายชล ไชยวงศ์วัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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MECHANISM OF SEX STEROID HORMONES IN GENE EXPRESSION CONTROL THROUGH LINE-1

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สายชล ไชยวงศ์วัฒนกุล : กลไกของฮอร์โมนเพศชนิดสเตียรอยด์ในการควบคุมการแสดงออกของยีนผ่านทางไลน์-1 (MECHANISM OF SEX STEROID HORMONES IN GENE EXPRESSION CONTROL THROUGH LINE-1) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ปฐมวดี ญาณทัศนีย์จิต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. นพ. อภิวัฒน์ มุทิรางกูร, ผศ. ดร. วิโรจน์ บุญรัตนกรกิจ, ดร. ศิษเฎศ ทองสิมา, 86 หน้า.

จากงานวิจัยก่อนหน้าพบว่าเบสซ้ำแบบกระจายยาว (ไลน์-1) มีความสำคัญในการควบคุมยีน ในการศึกษาครั้งนี้ได้ทำการเปรียบเทียบระดับ mRNA ของยีนที่มีไลน์-1 กับการศึกษาที่ใช้ฮอร์โมนเพศชนิดสเตียรอยด์ด้วยสถิติไคสแควร์ โดยใช้ข้อมูลการแสดงออกของยีนจากฐานข้อมูล NCBI จากการวิเคราะห์ผลของฮอร์โมนทั้ง 3 ชนิด พบว่ามีความเกี่ยวข้องกับการแสดงออกของยีนที่มีไลน์-1 โดยผลของฮอร์โมนเอสตราไดออล (E2) 100 นาโนโมลาร์ (nM) พบว่ามีความเกี่ยวข้องกับการแสดงออกที่เพิ่มขึ้นของยีนที่มีไลน์-1 ในเซลล์มะเร็งเต้านม (MCF-7 เซลล์ไลน์) ที่เวลา 3, 6 และ 12 ชั่วโมง ตามลำดับ (p-value = 6.38E-07, OR (95%CI) =1.43 (1.24-1.64); p-value =4.50E-13, OR (95%CI) 1.59(1.40-1.81) and p-value =1.18E-15, OR (95%CI) 1.64(1.45-1.85)) ต่อมาเป็นผลของโปรเจสเทอโรน (PG) ขนาด 10 นาโนโมลาร์ ที่ 16 ชั่วโมง ในเซลล์มะเร็งเต้านม (T-47D เซลล์ไลน์) พบว่ามีความเกี่ยวข้องกับการแสดงออกที่ลดลงของยีนที่มีไลน์-1 (p-value =9.53E-06, OR (95%CI) 1.65(1.32-2.06)) สำหรับผลของไดไฮโดรเทสโทสเทอโรน (DHT) ขนาด 10 นาโนโมลาร์ ที่ 16 ชั่วโมง ในเซลล์มะเร็งต่อมลูกหมาก (LNCaP เซลล์ไลน์) พบว่ามีความเกี่ยวข้องกับการแสดงออกของยีนที่มีไลน์-1 ลดลงเช่นกัน (p-value =3.81E-14, OR (95%CI) 2.01(1.67-2.42)) จากการศึกษาใน MCF-7 เซลล์ไลน์ พบว่ามีความสอดคล้องกับข้อมูลที่ได้จากการวิเคราะห์จากฐานข้อมูลดังกล่าวโดยพบว่าเอสตราไดออลที่ความเข้มข้น 100 นาโนโมลาร์ สามารถเหนี่ยวนำการแสดงออกเพิ่มขึ้นของยีนที่มีไลน์-1 ที่เวลา 12 ชั่วโมง สำหรับผลของโปรเจสเทอโรนพบว่าการแสดงออกของยีนที่มีไลน์-1 ใน T-47D เซลล์ไลน์ แสดงแนวโน้มไม่สม่ำเสมอดังผลวิเคราะห์ข้างต้น และเนื่องจากมีความผิดพลาดเกิดขึ้นกับ LNCaP เซลล์ไลน์ ดังนั้นจึงไม่ได้ศึกษาต่อถึงผลของโปรเจสเทอโรนและ ไดไฮโดรเทสโทสเทอโรน ผลจากการศึกษาการแสดงออกของยีนที่มีไลน์-1 โดยใช้สารแย่งจับกับตัวรับเอสโตรเจน (tamoxifen) สนับสนุนผลของ E2 ที่มีต่อการแสดงออกของยีนที่มีไลน์-1 โดยพบว่ามีผลทำให้ทั้งจำนวน mRNA ของไลน์-1 และยีนที่มีไลน์-1 ใน MCF-7 เซลล์ มีการแสดงออกลดลง นอกจากนี้เป็นที่น่าสนใจว่าจำนวนโพรบที่ตรวจสอบในยีนที่มีไลน์-1 และยีนแสดงออกมากขึ้น มีการเพิ่มจำนวนมากที่บริเวณหลังตำแหน่งไลน์-1 เทคนิค RNA sequencing จึงถูกใช้เพื่อยืนยันการเพิ่มขึ้นของรูปแบบ mRNA ของยีนที่เพิ่มขึ้นหลังตำแหน่งไลน์-1 ยิ่งไปกว่านั้นความสำคัญของบริเวณเข้าจับของโมเลกุลเอสโตรเจนและตัวรับบนดีเอ็นเอ (estrogen response element: ERE) ในกระบวนการเหนี่ยวนำยีนโดยเอสตราไดออล ถูกวิเคราะห์โดยการเปรียบเทียบยีนที่มีการแสดงออกเพิ่มขึ้นจากการศึกษาใช้เอสตราไดออล ระหว่างกลุ่มของยีนที่มีไลน์-1 ซึ่งมี ERE และไม่มี ERE ผลการศึกษาพบว่า ERE อาจไม่มีส่วนเกี่ยวข้องโดยตรงในกลไกของเอสตราไดออลในการเหนี่ยวนำการแสดงออกของยีนที่มีไลน์-1 จากการวิเคราะห์ข้อมูลโดยใช้ฐานข้อมูลการแสดงออกเพิ่มขึ้นของยีนที่ถูกเหนี่ยวนำด้วยเอสตราไดออลพบว่า c-fos ซึ่งอยู่ในกลุ่มยีนที่สามารถถูกกระตุ้นก่อนด้วยเอสตราไดออล (E2 early immediate gene) อาจมีความเกี่ยวข้องกับกระบวนการเพิ่มการแสดงออกของยีนที่มีไลน์-1 ซึ่งพิสูจน์โดย เทคนิค chromatin immunoprecipitation (ChIP) พบว่า c-fos มีความเกี่ยวข้องโดยมีบริเวณเข้าจับที่บริเวณ 5’ โพรโมเตอร์ของไลน์-1 และเข้าจับที่เวลา12 ชั่วโมง การค้นพบกลไกเหล่านี้แสดงถึงความรู้ใหม่ของฮอร์โมนเพศโดยเฉพาะเอสตราไดออล ในการควบคุมการแสดงออกของยีนโดยผ่านทางไลน์-1

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SAICHON CHAIWONGWATANAKUL: MECHANISM OF SEX STEROID HORMONES IN GENE EXPRESSION CONTROL THROUGH LINE-1. ADVISOR: ASST. PROF. PATTAMAWADEE YANATATSANEEJIT, Ph.D., CO-ADVISOR: PROF. APIWAT MUTIRANGURA, M.D., Ph.D., ASST. PROF. VIROJ BOONYARATANAKORNKIT, Ph.D., SISSADES TONGSIMA, Ph.D., 86 pp.

Long interspersed elements-1s (LINE-1s or L1s) was shown to play an important role in gene regulation. We further investigated the role of LINE-1s in gene regulation when the genes are under influence of hormones. The regulated mRNA levels of genes containing LINE-1 from the sex hormones treated experiments (available from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo)), gene expression database, was compared by chi-square. From the analyzed results, three types of sex hormones are strongly associated with expression of genes containing LINE-1. Firstly, in particular, 100 nM estradiol (E2) was associated with up-regulated genes containing LINE-1 at 3, 6 and 12 incubating hours, respectively (p-value = 6.38E-07, OR (95%CI) 1.43(1.24-1.64); p-value =4.50E-13, OR(95%CI) 1.59(1.40-1.81) and p-value =1.18E-15, OR(95%CI) 1.64(1.45-1.85)). For progesterone, we found that 10 nM of PG at 16 hours was related to down-regulated genes containing LINE-1 (p-value =9.53E-06, OR (95%CI) 1.65(1.32-2.06)). Lastly, the 10 nM of dihydrotestosterone (DHT) was shown to be associated with lower expression of genes containing LINE-1 (p-value =3.81E-14, OR (95%CI) 2.01(1.67-2.42)). The similar results in vitro and from database analysis were shown in 100 nM E2 which can induce the increasing of LINE-1s and also in genes containing LINE-1 expression after 12 hours for incubating time in breast cancer cells (MCF-7 cell lines). In contrast for PG, LINE-1s expression in breast cancer cells (T-47D cell lines) were not shown to have stable trends as expected. However, the influence of E2 to the expression of gene containing LINE-1 was supported by estrogen antagonist (Tamoxifen) experiment. Lower expression in both LINE-1s and genes containing LINE-1 was found after treating samples with Tamoxifen. On the other hand, the number of up-regulated probes were found higher at after LINE-1 position. Whole RNA sequencing using Hiseq 2000 was used to confirm the increasing of LINE-1 isoform which are produced by LINE-1 transcription in the E2 treated cells. Moreover, the role of ERE in E2 inducing genes pathway was analyzed by overlapping the genes which are up-regulated in E2 experiments with genes containing LINE-1. The results showed that ERE might not play a major role in E2 inducing transcription in genes containing LINE-1. Then, analyzing data from microarray expression was shown the association of estrogen early immediate genes (c-fos) which was related in the increasing of gene containing LINE-1 expression process. Chromatin immunoprecipitation (ChIP) was used to demonstrate this association and ChIP results showed the binding site of this transcription factors at 5' LINE-1 promoter. The relate function of c-fos was found at 12 hours for binding time at LINE-1 promoter. These findings indicate the new route of sex hormones in using LINE-1 to control gene expression.

Field of Study: Biological Sciences
Academic Year: 2015

Student's Signature .....
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CONTENTS

Page

Page

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

E2 = 17β-Estradiol

TAM = 4OH-Tamoxifen

ER = Estrogen receptor

HRE = Hormone responsive element

ERE = Estrogen responsive element

PG = progesterone

PRLR = Prolactin receptor

PGR = Progesterone receptor

ATR = Ataxia telangiectasia and Rad3 related

SINE = Short interspersed element

LINE-1 = Long interspersed element-1

RDX = Radixin

PHLDB2 = Pleckstrin homology-like domain, family B, member 2

MGST1 = Microsomal glutathione transferase 1

ALPK1 = Alpha Kinase 1

CDKN2B-AS1 = CDKN2B Antisense RNA 1

NAV2 = Neuron Navigator 2

NEK1 = NIMA-Related Kinase 1

NREP = Neuronal Regeneration Related Protein

RGS6 = Regulator Of G-Protein Signaling 6

RUNDC3B = Regulator Of G-Protein Signaling 6

c-fos = Cellular oncogene Fos

C/EBP = CCAAT-enhancer-binding protein

Chapter 1 Introduction

Eukaryotic gene expression was controlled at the initiation of transcription level. Cellular transcription is controlled by several complex interaction which compose of the signal transduction pathways, general and specific transcription factors, chromatin remodeling proteins and RNA polymerase (Ahmad and Kumar, 2011; Lodish et al., 2004). A large number of target genes were provided in common control by regulatory transcription factor that have several classes for transcription. These are composes the basal factors binding at the start point and TATA box. The transcription factor which act as activator can bind in promoter or enhancer site meanwhile co activator do not bind directly at DNA but do the connection between activators and basal apparatus (Lodish et al., 2000).

Controlling of gene expression by sex steroid hormones

Steroid hormones are known as stimulation on cell growth, development, differentiation, and homeostasis. These effects occur via their receptors, which act as hormone-dependent transcription factors. Steroid receptors can identify the specific hormone response elements in promoter area of target genes by ligand binding. Then the effect on transcription via co-activating or co-repressing complex that play direct interaction with activation function of receptor (Jenster et al., 1997 and Boonyaratanakornkit, 2012). Binding of hormone to the specific receptor will provide an activated form of receptor and turning into a transcriptional activator. Promoter activation and transcriptional initiation will occur when the steroid-receptor complex binds to the enhancer and then regulate a wide set of target genes (Lodish et al., 2004). Hormone response elements (HREs), the specific DNA sequences in target genes, are one of the most important regions of nuclear receptors for regulating transcription. HREs can be found in 5'-flanking region of the target genes, near to the core promoter and also in enhancer regions several kilobases upstream of the transcriptional initiation site (Aranda and Pascual, 2001). However, it's also found the binding of steroid receptors to negative HREs which can repress gene expression. Thus, steroid receptors

are the sequence-specific transcription factors because they interact at further upstream region of specific sequence. This mechanism is attract RNA polymerase II in promoters' transcribed (Quigley et al., 1995).

Sex hormone receptor (sex steroid receptors) is classified in homogenous subfamily. This subfamily is included estrogen receptor, estrogen-related receptors and classical steroid receptors (androgen receptor (AR), Mineralocorticoid receptor (MR), progesterone receptor (PR) and Glucocorticoid receptor (GR)) (Laudet, 1997). These receptors are interacted with specific sex hormones. First, ER (ERα, ERβ) binds estrogen like estradiol, estrone and estriol. Second, PR (PR-A, PR-B) binds progesterone and other progestogens. Third, AR binds androgens like testosterone and dihydrotestosterone (Maggiolini and Picard, 2010). It is well known that most of steroid hormones' biological effects exert via their receptors at the genes regulation level (Stanisic et al., 2010). The important function through ERs are included the control of female reproductive system, proliferation and differentiation especially in breast epithelium (Heldring et al., 2007). Level of endogenous estrogen is the primary factor that effective to development and progression in breast cancer (Jensen et al., 2010). Estradiol also is found to stimulate the growth through direct up-regulation of c-myc, which controls several cell cycles relating signaling gene in human breast cancer cell line (MCF-7) (Schraml et al., 1999). For result of the other steroid hormone, progesterone provides the strong evidence that associated in ovarian carcinogenesis (Liu et al., 2009) In 1995, Quigley and colleges found that the biological effect of androgens were mediated through AR. Recent data also confirmed the previous result that the dysregulation of AR activity and altered expression or binding of AR co-regulators has been found in development of prostate cancer (Bhasin and Jasuja, 2009; Chmelar et al., 2007).

The influence of retrotransposon elements in their host genes

The other mechanism that can control transcription of gene is driven from retrotransposons. Retrotransposons are dispersing all over the chromosome and effect to the regulation of human host gene's transcription (Matlik et al., 2006). Long interspersed element-1 (LINE-1 or L1) is one type of repetitive sequences in non-LTR

retrotransposons type which present more than 500,000 copies in human genome (Lander et al., 2001). Approximately 79% of genes in human transcription unit are found at least one segment of LINE-1s (Han et al., 2004). This indicates that LINE-1s might have some pathway which correlate to gene expression control. There are many reports about the relationship of LINE-1s in the spreading of X inactivation. Hypomethylation of intragenic LINE-1s was found in down-regulated genes for cancer and also in the genes during early embryogenesis in human and mouse (Aporntewan et al., 2011; Ngamphiw et al., 2014). The previous report were shown relationship of genes containing LINE-1 in various regulatory mechanism such as cells differentiation control, cell proliferation, and hormonal response mechanism. (Wanichnopparat et al., 2013). These evident were suggested that gene containing LINE-1 may associated to sex steroid inducing regulation pathway.

Up to now, there is no report about the relationship between intragenic LINE-1 insertion genes and sex hormones. This is the first study that can provide more function and mechanism of both LINE-1 and sex hormones in human genes expression control. Here, not only the relationship between intragenic LINE-1 insertion genes and sex hormones but also their controlling gene expression mechanism were studied in cancer cell lines. Objectives of this study are including

1. To determine sex steroid hormones regulation of LINE-1s expression.

2. To determine whether a subset of sex steroid regulated genes is mediated through sex steroid hormone induced LINE-1s expression.

3. To determine how sex steroid hormone regulated LINE-1s expression.

The advantage from this study is understanding the genes containing LINE-1 expression which were initiated by sex hormones in cancer cells.

Chapter 2 Literature reviews

Sex steroid hormones

Steroid hormones are known as stimulator of cell growth, development, differentiation and homeostasis. These effects occur via their receptors, which act as hormone-dependent transcription factors. Sex hormone synthesis is controlled by the release of hypothalamic gonadotropin-releasing hormone (GnRH) from pituitary gland. The stimulating of GnRH cause the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Target cells of LH were included leydig cells in male and theca cells in females (Mortimer and Yeo, 1976). The binding of these target cells can increase the expression of steroidogenic acute regulatory protein (StAR) which promotes the transfer of cholesterol to inner mitochondrial membrane (Homburg, 2014). Pregnenolone, produced principally in the adrenal gland, is act as pre-substrate for all sex steroid hormones. It will be converted from cholesterol and initiates steroidogenesis by cytochrome P450-mediated metabolic activation (Velarde, 2014).

Type of sex steroid hormones and target organ

1. Estrogen

Estrogen or 17β-estradiol (E2) is the primary female sex hormone. It is responsible for the development and regulation of the female reproductive system, and secondary sex characteristics. E2 refers to any substance, natural or synthetic, that mimics the effect of the natural hormone (Soltysik and Czekaj, 2013). In female, E2 is synthesized in the granulosa and theca cells of ovaries and corpus luteum. The granulosa cells are stimulated by LH to produce pregnenolone which can diffuse to adjacent theca cells. Then, theca cells will express 17α-hydroxylase and 3β-HSD which used for converting pregnolone to androstenedione via dehydroepiandrosterone (DHEA). DHEA can return to granulosa cells and converted to estrone by aromatase. After that 17β-HSD enzyme converts estrone to E2. In male, E2 and estrone are synthesized by converting from testosterone and androstenedione, respectively (Miller

and Auchus, 2011). Synthetic E2 are useful in a part of some oral contraceptives in E2 replacement therapy for postmenopausal women. E2 is synthesized in all vertebrates (Ryan KJ, 1982) as well as in some insects (Mechoulam et al., 2005). E2 synthesized in both vertebrates and insects suggesting that estrogenic sex hormones have an ancient evolutionary history. The three major naturally occurring forms of E2 in women are including estrone (E1), estradiol (E2), and estriol (E3). Another type of estrogen are called estetrol (E4) is produced only during pregnancy. Quantitatively, E2 circulation at lower levels than androgens were found in both men and women (Lombardi et al., 2001). While E2 levels are significantly lower in males compared to females, E2 also has important physiological roles in males. The actions of estrogen are mediated by the estrogen receptor (ER), a dimeric nuclear protein that binds to DNA and controls gene expression (Micevych and Kelly, 2012). The ER complex (E2 with ER) binds to specific DNA sequences which called estrogen response element (ERE) to activate the transcription of target genes. This identification was studied in estrogen-dependent breast cancer cell lines which were found in 89 genes (Lin et al., 2004). Since E2 enters to cells, its actions are dependent on the presence of the ER in the cell. The ER is expressed in specific tissues including the ovary, uterus and breast. The metabolic effects of estrogen in postmenopausal women has been linked to the genetic polymorphism of the ERs (Nelson and Bulun, 2001).

2. Progesterone (PG)

PG is an endogenous steroid hormone involved in the menstrual cycle, pregnancy, and embryogenesis of humans (Tekoa and Mary, 2010). PG is a crucial metabolic intermediate in the production of other endogenous steroids, including the sex hormones and the corticosteroids, and plays an important role in brain function as a neurosteroid (Baulieu and Schumacher, 2000). The result of its action occurs when binds to progesterone receptor (PR). PG is the most important hormone in the body, synthesize from pregnenolone by the action of 3β-HSD in corpusluteum or by placenta during pregnancy (Miller and Auchus, 2011). The synthesis of PG is vary with the menstrual cycle. It converts the endometrium to secretory stage and prepare the uterus for implantation. At the same time PG affects the vaginal epithelium and cervical

mucus, making it thick and impenetrable to sperm. If pregnancy does not occur, PG levels will be decreased and leading to menstruation. Normal menstrual bleeding is progesterone-withdrawal bleeding. The levels of PG will be low and leading to an ovulatory dysfunctional uterine bleeding in case of fail ovulation and the corpus luteum does not develop (Patel et al., 2014). In addition, PG inhibits lactation during pregnancy, so the low level of PG is one of the triggers for milk production. Sometimes progesterone were called as the "hormone of pregnancy" and it has many roles relating to the development of the fetus.

3. Androgen

Androgen (androgenic hormone) is natural or synthetic compound. This steroid hormone stimulates or controls the development and maintenance of male characteristics in vertebrates by binding to androgen receptors. The producing of adrenal androgen DHEA and androstenedione is in zona reticulata and zona fasciculata of adrenal cortex. DHEA will be converted to testosterone via intermediates androstenediol and androstenedione. For dihydrotestosterone (DHT) synthesis, androstenedione is converted to testosterone by 17β hydroxysteroid dehydrogenase. Then, testosterone will be conversed to DHT by the action of 5α-reductase in target tissues (Miller and Auchus, 2011). In generally, DHT plays an important role in almost of testosterone's biological action which includes the activity of the primary male sex organs and development of male secondary sex characteristics. DHT in the embryo life causes differentiation of penis, scrotum and prostate. In the late of life DHT contributes to male balding, prostate growth and sebaceous gland activity. The androgens function act as paracrine hormones required by sertoli cells to support sperm production. They are required for masculinization of the developing male fetus which include penis and scrotum formation. Under the influence of androgens, remnants of the mesonephron, the Wolffian ducts, develop into the epididymis, vas deferens and seminal vesicles. This action of androgens is supported by a hormone from sertoli cells, Müllerian inhibiting hormone (MIH). This hormone prevents the embryonic Müllerian ducts from developing into fallopian tubes and other female reproductive tract tissues in male embryos. Moreover, androgens promote the enlargement of skeletal muscle cells and

probably act in a coordinated manner to function by acting on several cell types in skeletal muscle tissue such myoblast (Sinha-Hikim I et al., 2004). Androgens increase not only in boys but also in girls during puberty (Zuloaga et al., 2008). In this phase, testosterone is converted to E2 by aromatase (P450aro) action. However, DHT is produced in peripheral target tissues of female, but low concentration in serum.

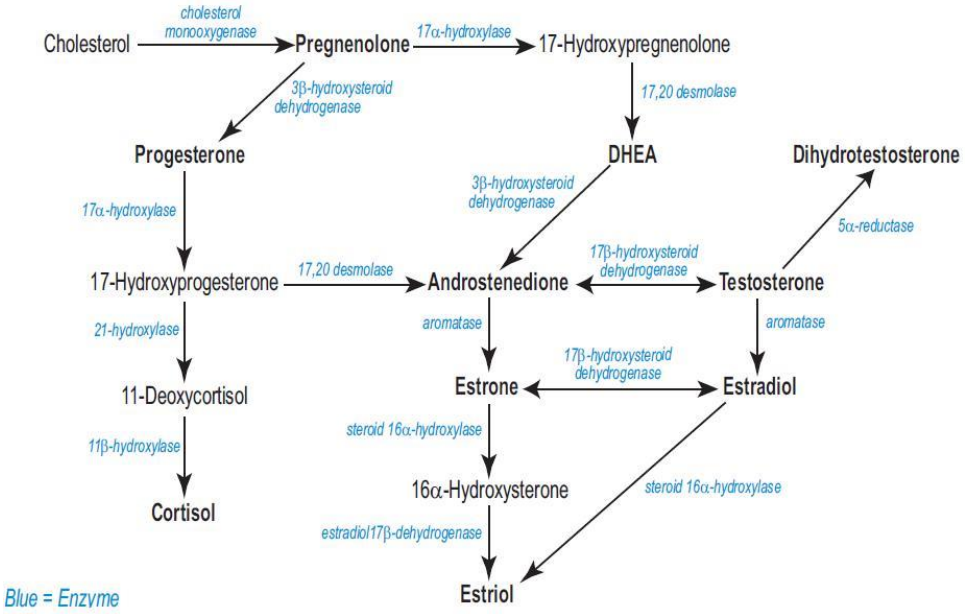


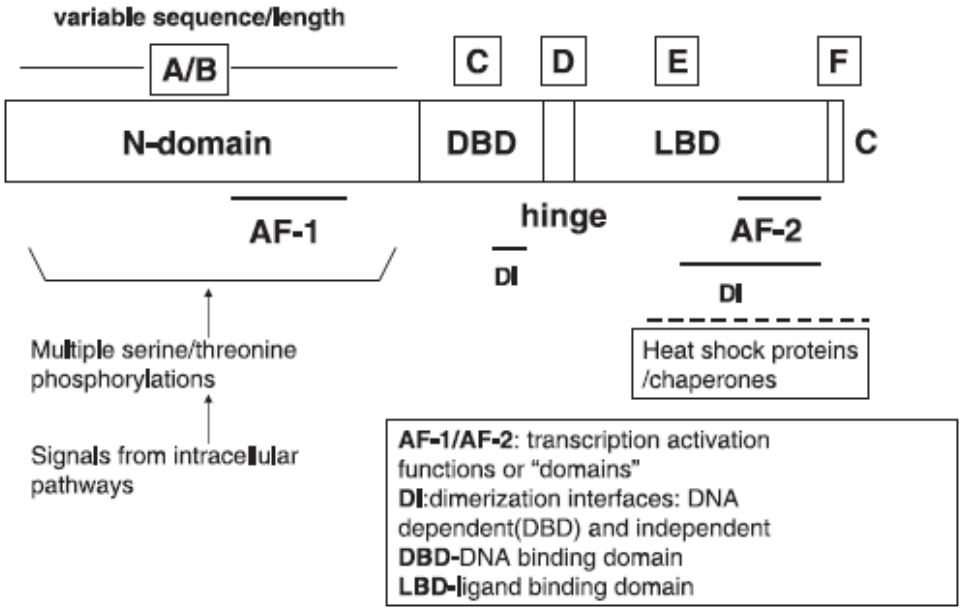
Figure1. Diagram of steroid hormone synthesis (Miller and Auchus, 2011)

Sex steroid receptor

It is well known that the most of steroid hormones' biological effects exert via their receptors at the genes regulation level (Thompson and Lippman, 1974). Sex steroid receptors are included nuclear receptors, class II receptors and orphan receptors. In nuclear receptors type, two molecules of each steroid receptor (homodimer) bound by the specific hormone and interacted at specific target DNA sequences. This sequences called hormone response elements (HREs). From this interaction, it can regulated genes transcription. For class II receptors, which act as heterodimers, can bind directly repeat HREs. These are including vitamin D, thyroid hormone, peroxisome proliferator, and retinoid receptors. In the last type, orphan

receptors act independent of the ligand as monomers on half-site HREs are including steroidogenic factor-1 and estrogen-related receptor (Wireman, 2007).

Figure2. Modular structure of steroid hormone receptors (Wireman, 2007).



N-domain represent NH2-terminus domain

C represent COOH-terminus domain.

The NH2-terminal region is composed the activation function area (AF-1). Dimerization interface are located in DNA binding region (DBD) and COOH-terminal region. AF-2 domain is located in ligand binding domain (LBD) which act as another transcription activation region It depend on their specific hormones (Figure 2) (Wireman, 2007).

Mechanism of sex steroid hormones

Genomic pathway

Steroid receptors can identify the specific hormone response elements in the promoter area of target genes by ligand binding. They effect the transcription via co-activator or co-repressor complex that play direct interaction with activation function of receptor (Jenster et al., 1997 and Boonyaratanakornkit, 2012). Binding of hormone to the specific receptor provides an activated form of receptor and turns into a

transcriptional activator. Promoter activation and transcriptional initiation occur when the steroid-receptor complex bind to the enhancer and then regulates a wide set of target genes (Lodish et al., 2004). HREs, the specific DNA sequences in target genes, are one of the most important regions of nuclear receptors for regulating transcription. HREs can be found in 5'-flanking region of the target genes, near to the core promoter and also in enhancer regions several kilobases upstream of the transcriptional initiation site (Aranda and Pascual, 2001). However, it has been found the binding of steroid receptors to negative HREs which can repress gene expression. Thus, steroid receptors are the sequence-specific transcription factors because interact at further upstream region of specific sequence and this mechanism can attract RNA polymerase II in promoters' transcribed (Quigley et al., 1995).

Non-genomic pathway

Some steroid hormone response is not only activate genomic pathway as described but non genomic pathway was also activated. Non-genomic regulation involves in the generation of intracellular second messengers and various signal transduction molecules including ion fluxes, cyclic AMPs and protein kinase pathways (Lösel and Wehling, 2003). Sex steroid hormones such as PG and E2 have been reported to regulate non genomic effect in cells of the reproductive organs (Pietras and Szego, 1975; Perret et al., 2001). The initial response of PG in non-genomic pathway involved in the inhibition of adenylate cyclase and the decreasing of cAMP (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981). The result of non-genomic pathway of PG shown in the activation of phosphatidylinositol 3- kinase (PI3K) and extracellular signal regulated kinase (ERK). Similar to PG, E2 has also regulate non genomic pathway in the cells of reproductive system. E2 was found to increase calcium level in endometrium and mature oocyte (Morley et al., 1992; Tesarik and Mendoza, 1995). E2 can bind to specific receptor on cell surface in endometrium, which indicate that ERs present at the cell surface (Pietras and Szego, 1977). The PI3K-Akt pathway has been shown to involve in this process which lead to the phosphorylation of eNOS and G protein coupled receptor (Wyckoff et al., 2001). However, non genomic effect

of E2 might not require ERs because the rapid activation of ERK pathway by E2 also occurs in ER-knockout mice (Toran-Allerand et al., 1999).

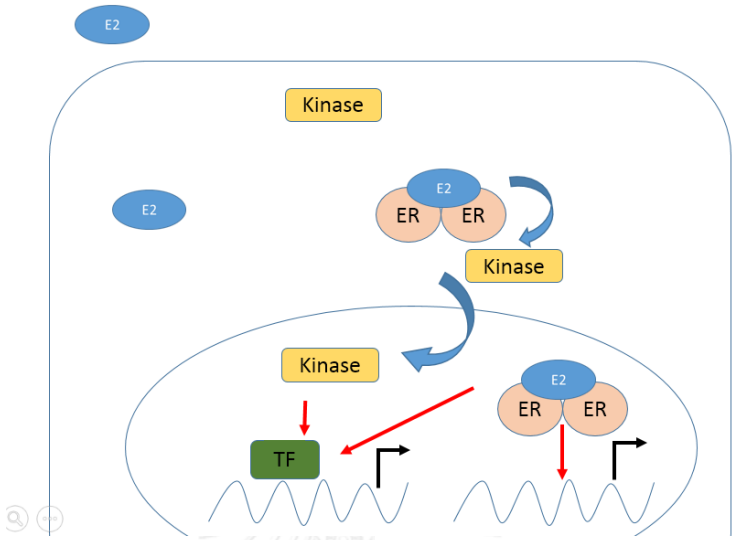


Figure 3. Genomic and non-genomic pathways of estrogen

Association of sex steroid hormones and cancers

The influence of sex steroid hormones almost effect to human physiological functions including activities and behaviors. Abnormal steroid hormone levels may result in variety of disorders including cancer. For generally of E2, it promotes cancers by cellular proliferation which stimulated through their receptor-mediated hormonal activity (Russo et al., 2006).

Whereas the cells with genetic mutations, they could become malignant, E2 triggers them to multiply out of control and form cancerous tumors. E2 also found to stimulate the growth through direct up-regulation of c-myc, which controls several cell cycles relating signaling gene, in human breast cancer cell line (MCF-7) (Schraml et al., 1999). Moreover, higher expression of ER-related genes were also presented in proliferative-related genes in breast cancer such as CCNB1, MKI67 and MYBL2 (Sorlie et al., 2003). For result of the other steroid hormone, PG provides the strong evidence that

associated in ovarian carcinogenesis (Liu et al., 2009). In PR-regulated genes have been shown the association with tumor progression towards aggressive tumor phenotype (Lange and Yee 2014). In 1995, Quigley and colleges were found that the biological effect of androgen was mediated through AR. Recently data also confirmed the previous result that the dysregulation of AR activity and altered expression or binding of AR co-regulators has been found in development of prostate cancer (Bhasin and Jasuja, 2009; Chmelar et al., 2007). Moreover, testosterone levels in serum have been found to be associated with advanced or high-grade prostate cancer (Takashi et al., 2009).

The Initiate of transcription and gene expression control

In bacteria, transcription begins with the binding of RNA polymerase to the promoter in DNA. The cooperation of RNA polymerase, a core enzyme, consisting with five subunits: 2 α subunits, 1 β subunit, 1 β' subunit, and 1 ω subunit. For the transcription initiation, the core enzyme is associated with a sigma factor (σ factor) that aids in finding the appropriate -35 and -10 base pairs upstream of promoter sequences (Mohamed et al., 2003). The holoenzyme was form when sigma factor and RNA polymerase combine. Then, they transcribed DNA template strand. Transcription initiation is more complex in eukaryotes. Eukaryotic RNA polymerase does not directly recognize the core promoter sequences. Instead, a group of transcription factors protein mediate the binding of RNA polymerase and the initiation of transcription were recruited. After transcription factors are attached to the promoter, the RNA polymerase can bind. Then the forming a transcription initiation complex will be occurred.

In eukaryotes, transcription occur within the membrane-bound nucleus. Then, the initial transcript is modified before it is transported from the nucleus to the cytoplasm for translation at the ribosomes. The initial transcript in eukaryotes has coding segments (exons) alternating with non-coding segments (introns). Before the mRNA leaves the nucleus, the introns are removed from the transcript by a process called

RNA splicing (Figure 4). Next, non-coding "caps" and "tails" are added to the ends of the transcript. These extra nucleotides protect the mRNA from the attack by cellular enzymes and aid in recognition by the ribosomes.

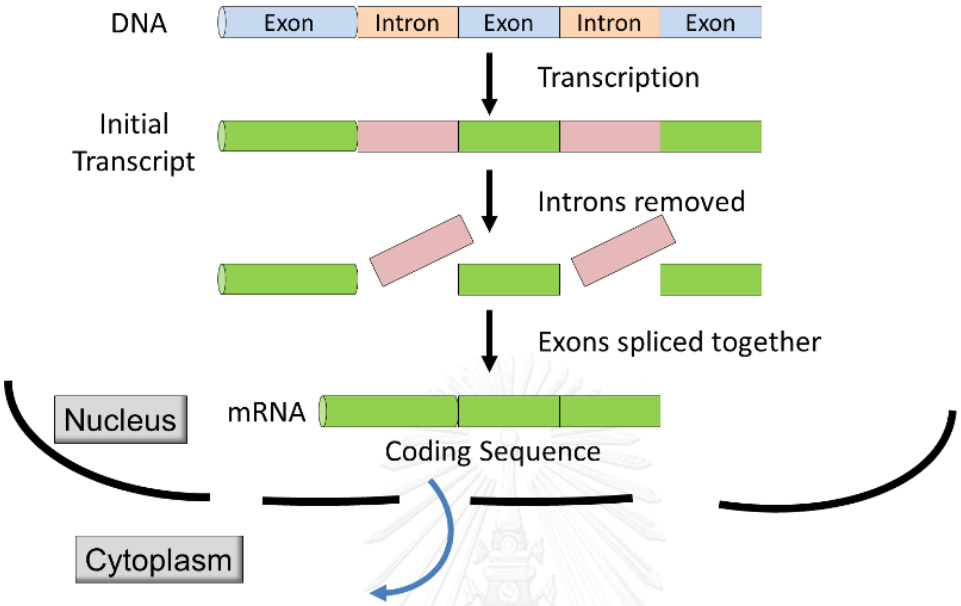


Figure 4. Transcriptional process in eukaryotes
(Source: http://unmug.com/category/biology/organisation-control-of-genome)

Gene expression can be influenced by signals from other cells. There are many examples in which a signal molecule (e.g., a hormone) from one cell binds to a receptor protein on a target cell. This can initiates a sequence of biochemical changes (a signal transduction pathway) that result in changes within the target cell. These changes can increase or decrease transcription as illustrated in the figure 5.

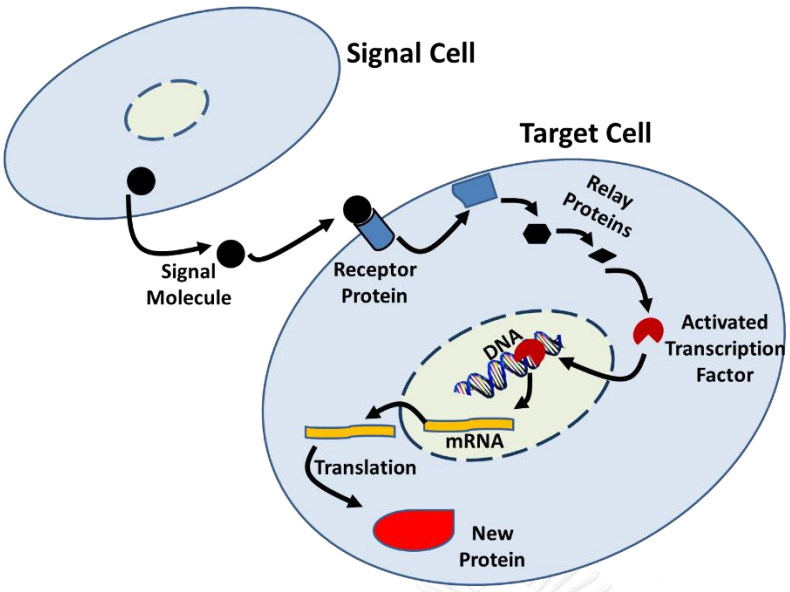


Figure 5. Signal transduction pathway to initiate the transcription.
(Source: http://sites.saschina.org/emily01px2016/2014/11/23/a-variety-of-intercellular-and-intracellular-signal-transmissions-mediate-gene-expression)

Repetitive sequences

Repeated sequences are patterns of DNA that occur in multiple copies throughout the genome. DNA sequence which can change the position within a genome called transposable element (TE) or transposon. Sometimes, TE creating or reversing mutations and altering genome size of the cells.

1. Tandem repeats

Tandem repeats occur in DNA when a pattern of one or more nucleotides is repeated and the repetitions are directly adjacent to each other. Mini-satellite is 10 and 60 nucleotides are repeated. Those with fewer are known as microsatellites or short tandem repeats. When exactly two or three nucleotides are repeated, it is called a dinucleotide or trinucleotide repeat (Oki et al., 1999). A variable number tandem repeat (VNTR) is the repeat unit copy number which variable in the population. Tandem repeat describes a pattern that helps to determine an individual's inherited traits.

2. Interspersed repeats

This repeats are found in all eukaryotic genomes. Certain classes of these sequences propagate themselves by RNA mediated transposition. They have been called retrotransposons which can dispersed widespread in eukaryotic genomes, and they constitute 25–40% of most mammalian genomes. Some types of interspersed repetitive DNA elements allow new genes to evolve by uncoupling similar DNA sequences from gene conversion during meiosis (Schimenti and Duncan, 1984). There are 2 main types in this class.

2.1 Short Interspersed Elements (SINEs)

This element is a short DNA sequences (<500 bases) which distributed throughout the non-centromeric regions of genome (over 100,000 copies per genome) (Weiner et al., 1986). SINEs present about 11% of the human genome (Richard and Mark, 2009). The most common of SINEs in primates is Alu. This elements are approximately 350 base pairs long, do not contain any coding sequences, and can be recognized by the restriction enzyme Alu I. The distribution of these elements has been implicated in some genetic diseases and cancers (Chenais, 2013).

2.2 Long Interspersed Elements (LINEs)

LINEs are a group of genetic elements that are found in large numbers in eukaryotic genomes, comprising 17% of the human genome. LINEs are composed open reading frames (ORFs) followed by a 3' A-rich region having 20,000 to 50,000 copies per genome (Hutchison et al., 1989). The active mobile DNAs in humans are the autonomous LINE-1s (L1s) retrotransposon. The LINE-1 life cycle composed of three steps. The first step is transcription of a genomic LINE-1 into RNA, which is mediated by RNA polymerase II from an internal L1 promoter. Transcription from an internal antisense LINE-1 promoter may also occur. In the second step, the RNA is translated into two LINE-1-encoded proteins: ORF1p, an RNA-binding protein, and ORF2p, a protein with reverse transcriptase and endonuclease activities. These proteins associated with the LINE-1 transcription, and resulting in ribonucleoprotein (RNP) complexes which then transferred to the nucleus. The third step is termed target-primed reverse transcription (TPRT). In this step, ORF2p cleaves the target DNA (often

at a 5′-TTTTAA-3′ consensus sequence) and uses the 3′ hydroxyl group to prime the reverse transcription reaction. Synthesis of the second strand and resolution of the structure are still poorly understood. LINE-1 life cycle can generate DNA breaks and transcriptome effects to cell host proteins that mediate DNA repair (Figure 6).

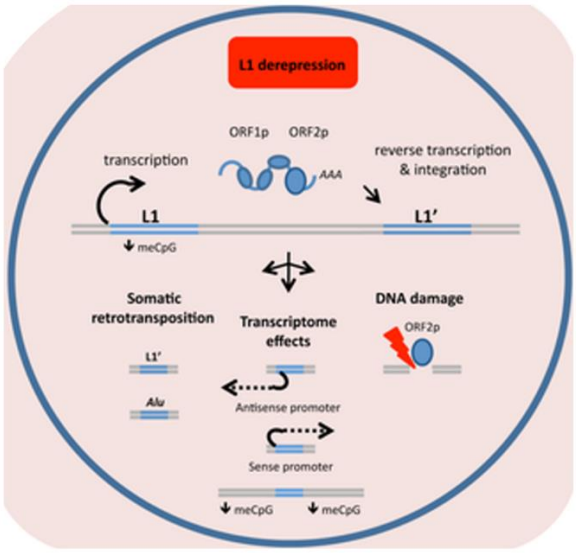


Figure 6. Show LINE-1 retrotransposon life cycle (Rodić and Burns, 2013).

Source:

https://figshare.com/articles/_DNA_methylation_and_related_mechanisms_inhibit_LINE_1

Intragenic LINE-1 regulates gene expression

There are two classes of LINE-1s which present in our genome. These are included intragenic and intergenic LINE-1. The different type of LINE-1 are distinguished depending on the location of LINE-1s sequences. Intragenic LINE-1 means LINE-1s which located within genes meanwhile intergenic LINE-1s were located between genes (Figure 7). Intragenic LINE-1 were found repressed gene expression in cancer (Aporntewan et al., 2011).

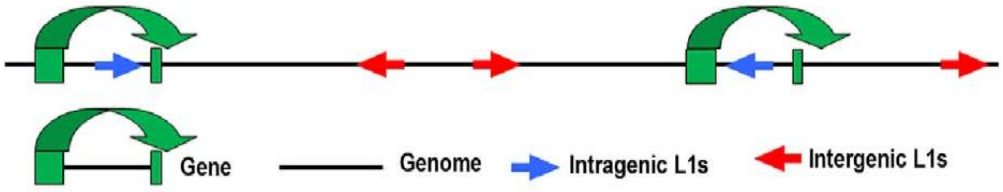


Figure 7. Two classes of LINE-1s. Intragenic LINE-1s represented by blue arrows and intergenic LINE-1 represented by red arrows (Aporntewan et al., 2011).

There are two ways for the LINE-1 promoter to produce unique RNA sequences. The 5′ UTR of LINE-1 is a promoter that transcribes in both the forward and reverse directions (Matlik et al., 2006; Weber et al., 2010; Speek, 2001 and Wolff et al., 2010). For the transcription in forward direction, LINE-1 promoter produces LINE-1 RNA. However, the poly-A addition signal of LINE-1 does not always function. Moreover, many LINE-1 transcripts can continue to the end of the LINE-1 sequence, therefore resulting in 3′ transduction (Moran et al., 1999 and Rangwala et al., 2009). These transduction sequences are unique RNA sequences generated by the LINE-1 promoter. On the other hand, LINE-1 5′ transduction that occurs by reverse transcription also produce unique RNA sequences. A large number of these transduction sequences have been reported (Rangwala et al., 2009); however, there are currently only two examples that prove that these sequences are increased by LINE-1 hypo methylation (Weber et al., 2010; Wolff EM et al., 2010; Aporntewan et al., 2011).

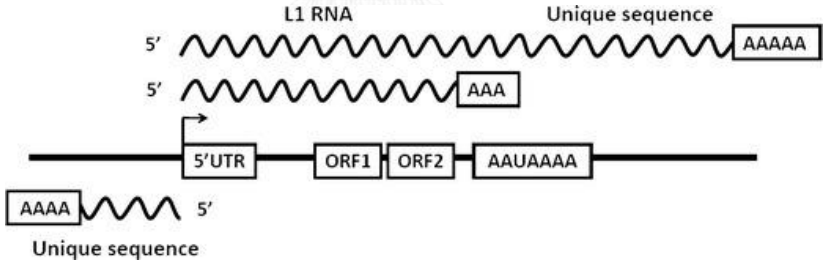


Figure 8 Two types of unique RNA sequences which produced from LINE-1 (Kitkumthorn and Mutirangura, 2011).

Intragenic LINE-1 regulation of host gene expression was revealed by the finding that in vitro insertion of a full-length LINE-1 disrupted host gene expression (Han et al., 2004). In vivo, this gene regulation is related to LINE-1 methylation levels (Aporntewan et al., 2011). LINE-1 methylation levels were reduced by chemical treatment or by carcinogenesis. The significant number of genes containing LINE-1s were repressed correlated with the intragenic LINE-1 methylation level. The role of LINE-1 methylation is to prevent the formation of a pre-mRNA–LINE-1–RNA complex. This complex is

formed, then the RISC protein AGO2 will bind and prevent mRNA production (Aporntewan et al., 2011).

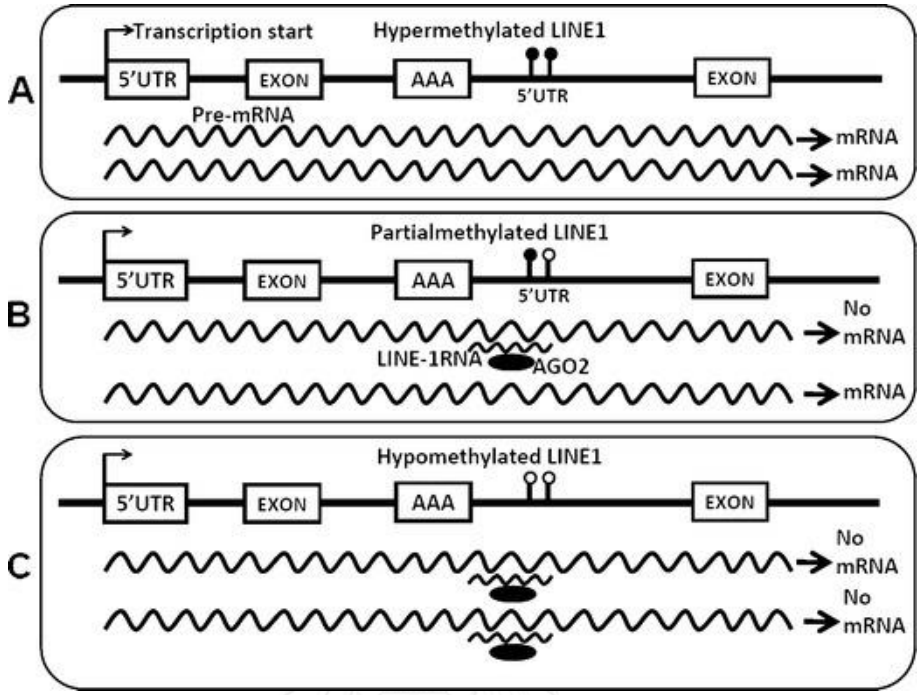


Figure 9. Intragenic LINE-1 can regulate gene expression. LINE-1 RNA is produced when the methylation of the LINE-1 5′ UTR is reduced. The LINE-1 RNA–pre-mRNA complex is bound by AGO2, and mRNA production is inhibited (Kitkumthorn and Mutirangura, 2011). .

Association of Intragenic LINE-1s to cancers

In some cases, transcription is initiated within an LINE-1 to form a chimeric mRNA that contains L1 sequence (sense or antisense) and downstream exons of a host gene. In fact, in normal mouse tissues, transcription is initiated frequently within LINE-1 sequence, as Faulkner et al. determined by sequencing the 5′-most nucleotides of RNAs from both normal and neoplastic tissues, using cap-analysis gene expression tag technology (CAGE) (Faulkner et al., 2009). Transcription initiation often occurs within 5′ L1 sequence, which contains both sense and antisense promoter activities. In human cancers, aberrant expression of chimeric transcripts may play important roles in tumor genesis. The researchers have noted transcript variants initiated by LINE-1 antisense promoters in bladder carcinoma (Wolff et al., 2010), chronic myeloid leukemia (CML)

(Bollati et al., 2009), esophageal adenocarcinoma (Lin et al., 2006), and breast carcinoma (Cruickshanks and Tufarelli, 2009).

Chapter 3 Materials and methods

1. Bioinformatics analysis

Identification of up-or down-regulated expression analysis

The intersection of microarray expression data of sex hormones regulated gene, which is compatible with Gene Expression Omnibus (GEO) and genes containing LINE-1 library were done. Connection up-or down-regulation expression analysis of microarrays extension (CU-DREAM EX) is a bioinformatics program which is used to study the relation of genes expression from interested experiment and genes containing LINE-1. The CU-DREAM EX program and user guideline can be downloaded at http://pioneer.netserv.chula.ac.th/~achatcha/cu-dream. The means of mRNA levels in control and experimental groups from each experiment was performed by student's t-test. To evaluate the influence of LINE-1s to host gene expression, genes were divided into two categories, containing LINE-1 or not containing LINE1. The number of genes in each subset were compared by using chi-square test. Microarray expression of mRNAs from GEO data set (Edgar, 2002) was classified as up-or down-regulated and not up-or not down-regulated depending on the statistical significance determined by student's t-test. The 2x2 table was provided for frequency of occurrence. For odd ratio calculated, the number of up or down-regulated genes containing LINE-1 was divided by the number of not up-or not down-regulated genes containing LINE-1. Then, the number of up or down-regulated genes not containing LINE-1 was divided by the number of not up-or not down-regulated genes that did not contain LINE-1. An odd ratio of more than 1.0 indicated the association between effect of sex steroid hormones and genes containing LINE-1 expression. The expression of each gene was classified into four groups based on p-value. The genes containing LINE-1 which were either up or down regulated were included in group A. Meanwhile, genes not containing LINE-1 were included in group B. Group C was composed of non-significant up or down-regulated genes containing LINE-1. The last, group D, was composed of non-significant up or down–regulated genes not containing LINE-1. Percentages of up or down-regulated and not up or not down-regulated genes containing LINE-1 which

were induced by sex steroid hormones were analyzed. These were calculated by using the number of genes containing LINE-1 in up or down-regulated divided by the total number of up or down-regulated genes with and without LINE-1. Similarly, the number of not up or not down-regulated genes were divided by the total number of not up or not down-regulated genes with and without LINE-1.

Expression of genes containing L1s and classification of mRNA

Intragenic LINE-1s (from LINE-1 base) were categorized according to their genomic base in the NCBI Reference Sequence (RefSeq) annotation (Penzkofer et al., 2005) which was the same as previously reported (Aporntewan et al., 2011). Three experiments from public array expression were analyzed. Breast cancer cell lines which were selected in these studies include MCF-7 (for E2 study), T-47D (for PR study) and ZR-75-1 (for DHT study). Cancer cell lines were treated by E2 with 100 nM at 3, 6 and 12 hours (GSE11324, Carrol et al., 2006). For PR (GSE62243) and DHT (GSE61368) (Need et al., 2015) were also treated with 10 nM at 16 hours. GEO sample numbers (GSMs) in each experiment was separated into control (non treated hormone) and experimental groups (treated hormone). A significance level of 0.05 was used in each experiment. Genes containing LINE-1 lists were collected from L1Base (http://line1.bioapps.biozentrum.uniwuerzburg.de/l1base.php) (Penzkofer et al., 2005).

1.1 Candidate genes identification

The experiments in GEO Series (GSE) of sex steroid hormones were chosen for identify candidate genes in this study. These GSEs were intersected with the library of gene containing LINE-1 by CU-DREAM EX program. Then, the significant different of result in each intersection was selected. Student's t-test will be performed to calculate significant differences between control (non-treated) and treated cell with sex hormones in various times and doses. All p-values were obtained by two sided and p-values less than 0.05 were observed to statistical significant.

1.2 Element binding sequences analysis

The position of estrogen response element (ERE) and transcription binding sites of estrogen early immediate genes on intragenic LINE-1 were analyzed to determine the region of steroid hormone receptor and intragenic steroid hormone response elements (HREs) in gene containing LINE-1. IntragenicLINE-1 locations, gene containing L1s from LINE-1 Base (Penzkofer et al., 2005) also find and generate to UCSC genome browser format to retrieve the corresponding LINE-1s sequences. LINE-1 locations, gene names, the number of each response elements in those LINE-1s and the position and location of each response element related to LINE-1s location, "DNA-pattern" on the RSAT (Regulatory Sequence Analysis Tools) will be calculated to obtain the results of correspond element on each LINE-1. All candidate genes were collected from LINE-1 contain HREs genes that regulated with specific hormone. Sequence of ERE: NNGNCANNNTGNCNN was used for analyze. "N" represent guanine or adenosine or thymine or cytosine,

1.3 Specific estrogen early immediate gene analysis

To determine the specific estrogen early immediate genes that are related to estrogen induce gene expression mechanism, the binding region of transcription factors that act as estrogen early immediate gene were analyzed. Genes that contain the binding site of these estrogen early immediate genes were intersected with gene containing LINE-1 that up regulated after treated with estrogen by CU-DREAM EX program. The binding sequence region of transcription factors were include as following

c-jun#1 = TGACGTCA (Li et al., 2011)

c-jun#2 = TGASTCA (Li et al., 2011)

c-fos#1 = TGACTCA (Seldeen et al., 2009)

c-fos#2 = TGTSTCA (Seldeen et al., 2009)

E2F1 = TTTSSCGS (Zachariadis and Gorgoulis, 2009)

Cathepsin D = GTGCGTG (Krishnan et al., 1995)

Forkhead = ANRBAARBRN (Carroll et al., 2006)

AP-1 = TGACBCR (Carroll et al., 2006)

C/EBP = NNNTTRSNHMVNNN (Carroll et al., 2006)

Oct = NNNNRTGYAAAKNMN (Carroll et al., 2006)

1.4 Up-regulated probes quantity analysis

To determine the part that have important effect to the up regulated gene, the number of up regulated probes at both before and after LINE-1 position was calculated. All significant results of up regulated genes that were treated with estrogen from CU-DREAM EX calculation will be divided into two parts. The first part was started from transcription start site to LINE-1 position and the second part was started from LINE-1 position to poly A-tail of the gene structure. The number of up regulated probes of all up regulated gene was analyzed by CU-DREAM EX program.

2. Cell culture

The established human breast cancer cell lines which obtain from American Type Culture Collection (Rockville, MD), MCF-7 (ATCC: HTB-22TM), were all grown up with Phenol red fee DMEM medium supplemented with 10% FBS (Gibco® BRL, Life Technologies) at 37 °C in a humidified atmosphere (95% air: 5% CO2). The cell lines were passaged twice a week, and the medium was changed every other day. The cells, grown in culture flasks (CytoOne T225 flask, USA Scientific® .Inc), were harvested at 60-65% confluence using 0.05% Trypsin, 0.5 mM EDTA, and washed in a phosphate buffer saline (PBS). ERs in MCF-7 cell lines were checked by ESR1 primer ID 1941 (RTPrimerBD) before used in the experiments. For experiments, cell lines were grown in 6 wells plate about 70-80%. Then cells were switched to phenol red-free medium (HyCloneTM, Logan, Utah) with 5% chacoal stripped FBS (Sigma®, UK) and 1% antibiotic (Invitrogen, U.S.A.) at least 1 day, before treated either with 17-β estradiol (1- 250 nM; Sigma®, UK), or TAMOXIFEN, 4-HYDROXY-, (Z)- (estrogen antagonist) (100 nM; Merck®). For estrogen antagonist experiment, MCF-7 cells were treated with TAMOXIFEN for a half an hour, followed by 17-β estradiol.

3. RNA extraction and cDNA synthesis

Total RNA from MCF-7 cell lines were extracted by Trizol reagent (Invitrogen, Carlsbad, CA). RNA was treated with RNase-Free DNase Set (QIAGEN, Germany) to remove any contaminating DNA. To synthesize cDNA, Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, USA) was used. Concentration of 5 μg/12 μl DNA-free RNA in DEPC-treated water was performed and 0.5 μg oligo (dT) 18 primer (Fermentas) was used. The total RNA was incubated for 5 min at 70 °C and chilled on ice for 5 min. For reverse transcription, the mixtures were incubated with 200U RevertAidTM M-MuLV Reverse Transcriptase (Fermentas), 20 U RibolockTM Ribonuclease inhibitor (Fermentas) and 20 mM dNTPs at 42 oC for 60 min, followed by 95 oC for 5 min and finally cooled rapidly at 4 oC. To evaluate the amount of LINE-1 DNA contamination, RNA that had not been reverse transcribed was used as negative control.

4. DNA extraction

The cell pellet from MCF-7 cell line was washed twice in sterile PBS. One milliliter of the DNA extraction buffer with 10% SDS and proteinase K (0.5 mg/ml) was added to the cell pellets. The mixtures were then incubated at 50°C for two nights. A phenol-chloroform extraction was used to purify and desalt the digested cell pellets. After centrifuging at 4°C, 14000 g for 15 minutes, 10 M ammonium acetate and cold absolute ethanol were added to the upper aqueous phase for DNA precipitation. The precipitated DNA was washed with 70% ethanol. The air-dried DNA was then resuspended in Tris-EDTA-treated water.

5. Polymerase Chain Reaction (PCR)

Totally 10 μL of PCR master mixed was prepared in duplicate, containing 4μL of the recovered PCR product, 1x PCR buffer, 0.1μL of dNTP and 0.5U of Taq polymerase. The PCR steps were run at 95°C for 2min, 50°C for 1min and 72°C for 1min at 35 cycles.

6. Gel electrophoresis

The amplification products were identified by polyacrylamide gel electrophoresis (8% non-denaturing). The condition was used as 100 volt for 50 minute and stained with SYBR green nucleic acid stain (Sigma-Aldrich, St. Louis, Missouri). Distilled water was used as a negative control.

7. Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed the binding site of E2 early immediate genes (c-fos and C/EBP) on 5'LINE-1 promoter. The protocol was modified from previously research (Kongruttanachok et al., 2008; Boyd and Farnham, 1999). The processes were started by the cross-linking of protein and DNA. Followed by sonicating to shear DNA strands and preclear the samples by protein A. Then, add bead-attached antibodies to immunoprecipitate target protein. The chromatin fragments were immunoprecipitated with normal mouse IgG antibody as a negative control (Santa Cruz Biotechnology). Specific protein antibody c-fos and C/EBP (Santa Cruz Biotechnology) were used. Finally, antigen-antibody complexes were washed by low to high salt washing buffer and reverse crosslink by 5 M NaCl. To prove the binding site of these two proteins on intragenic LINE-1s, specific primers which located at c-fos and C/EBP binding sites in each candidate genes on the 5' end LINE-1 were designed as following.

PGR (c-fos binding site): forward primer 5'-CTT-TTC-TTC-TCC-CTG-CTC-CC-3'
reverse primer 5'-TGT-GAC-TGA-ATC-TGG-ATG-ATG-A-3'

PRLR (c-fos binding site): forward primer 5'-GCA-TCA-AAC-TTC-CCT-TCC-AA-3'
reverse primer 5'-CCC-ATG-ATG-GTT-TTT-CAT-CC-3'

PRLR (C/EBP binding site): forward primer 5'-CTT-ACT-TGG-GCT-CTG-TTC-GC-3'
reverse primer 5'-GCA-GAA-GGC-CTG-ACA-AAC-AT-3'

Then, the expected products were prove by polyacrylamide gel electrophoresis.

8. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative analysis of LINE-1 mRNA was performed by using the Light CyclerTM system (Roche Diagnostics) with the Fast-Start DNA Master SYBR Green I Light Cycler Kit (RocheDiagnostics). PCRs were run in triplicate with 20 µL reaction volumes containing 1x SYBR Green I PCR Buffer Mix, 5 mM MgCl2, 0.5 µM each primer, 1x FastStart Taq DNA Polymerase (Roche Diagnostics), and 50 ng of cDNA. The cycling conditions was performed for 40 cycles with an annealing temperature of 60 °C. For 5'LINE-1 RNA primers are composed 5'-GGCCAGTGTGTGTGCGCACCG-3' and 5'-CCAGGTGTGGGATATAGTCTCGTGG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression primers were served as reference gene (Forward: 5'-CAG-CCG-CAT-CTT-CTT-TTG-3', reverse: 5'-ATC-CAC-TTT-ACC-AGA-GTT-AA-3'). Primers for candidate genes expression were designed on exon that present up regulate probe. Forward and reverse primers are given below:

PGR forward: 5'-GCGCCCGAAACTTACATATTGATGACC-3'

PGR reverse: 5'-TACAGCATCTGCCCACTGACGTG-3'

PRLR forward: 5'-GAGAAGGCAGCCAACATGAAGG-3'

PRLR reverse: 5'-CATTCAGAAGGCAGGTGTTGAG-3'

ATR forward: 5'-GCGCCTAGCCTATCCTCAACAAGC-3'

ATR reverse: 5'-CCTTGCATCTGTTCACACGCATGGG-3'

The c-fos and C/EBP primers were used from previously reports (Seldeen et al., 2009; Carroll et al., 2006) to observe their expression. Melting curve analysis was performed for each reaction to exclude nonspecific PCR side products. Relative quantification by comparative CT method was used and referred to the $2^{-\Delta\Delta CT}$ method.

9. Reverse transcriptase PCR (RT-PCR)

To evaluate ERs, PRs expression, RT-PCR was performed with specific primer (Al-Hendy et al., 2006; Chevillard et al., 1996). by using MCF-7 and T47D cell lines (Al-Hendy et al., 2006; Shidaifat, 2009) Total RNA was extracted from cell lines using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The

RNA was treated with RNase-free DNaseI (Fermentas) to remove contaminating genomic DNA and with RiboLock™ Ribonuclease Inhibitor (Fermentas) to prevent degradation. To synthesize cDNA, 5 µg DNA-free RNA was dissolved in 12 µl of DEPC-treated water containing 0.5 µg oligo (dT) 18 primer (Fermentas). The RNA was incubated for 5 min at 70°C and chilled on ice for 5 min. Each sample was then incubated with 200U RevertAid™ M-MuLV Reverse Transcriptase (Fermentas), 20 U Ribolock™ Ribonuclease inhibitor (Fermentas) and 20 mM dNTPs for 1 hr at 42°C, followed by 10 min at 70°C and subsequent chilling on ice. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for internal control.

Chapter 4 Results

Comparing of mRNA regulation in genes containing LINE-1 from sex hormones treated experiments, which available from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo), were done by chi-square. Then, sex hormones influence to genes containing LINE-1 expression in cancer cells were evaluated. The different concentration in three types of sex hormones were included 100 nM estradiol (E2), 10 nM progesterone (PG) and 10 nM dihydrotestosterone (DHT). Up-regulate genes in 100 nM E2 treatment, were shown in all three incubating times. Interesting that the highest significant was shown at 12 hours when compared to 3 and 6 hours. The increasing of significant was followed by incubating times from 3 to 12 hours, respectively (p=6.38E-07, OR (95%CI)=1.43(1.24-1.64); p=4.50E-13, 1.59(1.40-1.81) and p=1.18E-15, 1.64(1.45-1.85) (Table1)). In contrast, no significant result (odd ratio less than 1) was found in down-regulate genes containing LINE-1 in all incubating times (Table 1). For the influence of PG to genes containing intragenic LINE-1 expression in T-47D cells. The concentration at 10 nM PG for 16 hours were observed and down regulation were shown (p= 9.53E-06, 1.65(1.32-2.06)). No significant result was shown in up-regulated genes (Table 1). Finally, both down and up-regulated genes were shown in 10 nM DHT which incubated in ZR-75-1 cells for 16 hours (p= 3.81E-14, 2.01(1.67-2.42); P=4.03E-02, 1.40(1.01-1.95) (Table 1)).

4.1 The regulation of LINE-1 expression by sex steroid hormones

From the preliminary results, experiment in vitro was done. Breast cancer cell lines (MCF-7) were used for studying the influence of E2 to intragenic LINE-1s expression compared to non-treatment. Frist, ER-alpha in MCF-7 cells were checked by RT PCR and compared to HN17 cells (negative control). The expected results were found. Product sizes of ER-alpha (108 bp) were presented only in MCF-7 cells, but not presented in HN17 cells. Product sizes of GAPDH (99 bp) (positive control) were shown

in both cell lines meanwhile non template cDNA were not present product sizes in both genes (Figure 10). Dose response of the concentration and incubating time points of E2 for inducing the highest intragenic LINE-1 expression were studied. First, 100 nM E2 was used for investigating the appropriate incubating time points. The incubating time points were as followed: 3, 6, 12 and 24 hours. The results were shown that 12 hours presented the highest LINE-1 expression when compared to non-treat and the rest incubating time points ($p=0.0137$) (Figure 11). Then, dose response for E2 concentration was done for proving the highest of intragenic LINE-1s expression. Concentration was used are as follow 10, 50, 100, 150 and 200 nM. The appropriate condition to induce the highest intragenic LINE-1 expression in breast cancer were present 100 nM E2 at 12 hours ($p=0.0012$) (Figure 12). This results were supported by bioinformatics data in preliminary (Table 1).

Moreover, the influence of various PG concentrations to intragenic LINE-1 expression were also studied. Concentration of PG were composed 1, 10 and 100 nM and treated for 3, 6 and 12 hours. Non stable trend of intragenic LINE-1 expression results were shown, especially at the same concentration but different incubating time points (Figure 13). Suggesting that PG may not only one activator for inducing intragenic LINE-1 expression. Then, for the influence of DHT to genes containing LINE-1 expression in LNCaP cells, there was problem about the transporting of this cell lines. So, from now on, only the influence of E2 to genes containing intragenic LINE-1 expression were shown.

To prove the inducing of up-regulated intragenic LINE-1 expression by E2, estrogen antagonist (Tamoxifen) was used. The various concentration of Tamoxifen composed 1μM, 150, 100, 50 and 10 nM. The lower expression of intragenic LINE-1s in Tamoxifen treated when compared to E2 treated samples were expected. Three concentrations of Tamoxifen were shown as the same trend for this condition (150, 100 and 50 nM) (Figure 14). Then, 100 nM E2 was selected in the next experiment. Tamoxifen (100 nM) was treated to MCF-7 cells for 30 minutes before incubated with 100 nM E2 for 12 hours. Significant decrease of LINE-1 expression was shown in Tamoxifen with E2 treated samples when compared to non-treat and 100 nM E2

treated cell (p=0.0001) (Figure 15). Moreover, significant three candidate genes containing LINE-1, which were up-regulated when incubating with 100 nM E2 for 12 hours, were selected. These genes had related function to cancer promoting which composed as

1. Prolactin receptor (PRLR): a receptor for the anterior pituitary hormone prolactin (PRL) (p=9.04E-05).

2. Progesterone receptor (PGR): inactive in stimulating c-Src/MAPK signaling on hormone stimulation (p=3.51E-05).

3. Ataxia telangiectasia and Rad3 related (ATR): a serine/threonine-specific protein kinase that is involved in sensing DNA damage and activating the DNA damage checkpoint, leading to cell cycle arrest (p=5.50E-04).

PRLR, ATR and PGR were shown the same trend with the increasing expression of intragenic LINE-1s after 100 nM E2 was treated when compared to non-treated (p= 0.0384, p= 0.0214 and p= 0.0208; figure 16, 17 and 18). In contrast, decreased of LINE-1s expression were found after Tamoxifen was treated (p=0.0382: ATR) (Figure 17). The same trend also shown in the sample which treated with 100 nM Tamoxifen and 100 nM E2 when compared to non-treated (Figure 16, 17 and 18). (p=0.0461: PRLR; p=0.0126: ATR and p=0.014: PGR).

Sex steroid hormones	GSE	Concentration (Incubating time)	Gene regulation	LINE-1	No LINE-1	Odd ratio	95% CI	p-value
Estrogen	11324	100 nM (3 hours)	Up	264	2923	1.43	Lower: 1.64	6.38E-07
			Not up	1037	16389		Upper: 1.24	
			Down	152	4269	0.47	Lower: 0.55	7.67E-19
			Not down	1149	15043		Upper: 0.39	
		100 nM (6 hours)	Up	358	3719	1.59	Lower: 1.81	4.50E-13
			Not up	943	15593		Upper: 1.40	
			Down	216	5592	0.49	Lower: 0.57	9.05E-22
			Not down	1085	13720		Upper: 0.42	
		100 nM (12 hours)	Up	415	4300	1.64	Lower: 1.85	1.18E-15
			Not up	886	15012		Upper: 1.45	
			Down	268	6467	0.52	Lower: 0.59	8.55E-22
			Not down	1033	12845		Upper: 0.45	
Progesterone	62243	10 nM (16 hours)	Up	57	1202	0.93	Lower: 1.22	6.03E-01
			Not up	1001	19634		Upper: 0.71	
			Down	91	1126	1.65	Lower: 2.06	9.53E-06
			Not down	967	19710		Upper: 1.32	
Dihydrotestosterone	61368	10 nM (16 hours)	Up	40	538	1.40	Lower: 1.95	4.03E-02
			Not up	1250	23612		Upper: 1.01	
			Down	139	1369	2.01	Lower: 2.42	3.81E-14
			Not down	1151	22781		Upper: 1.67	

Table 1. The relationship of genes containing LINE-1 expression and sex steroid hormones ($p<0.05$).

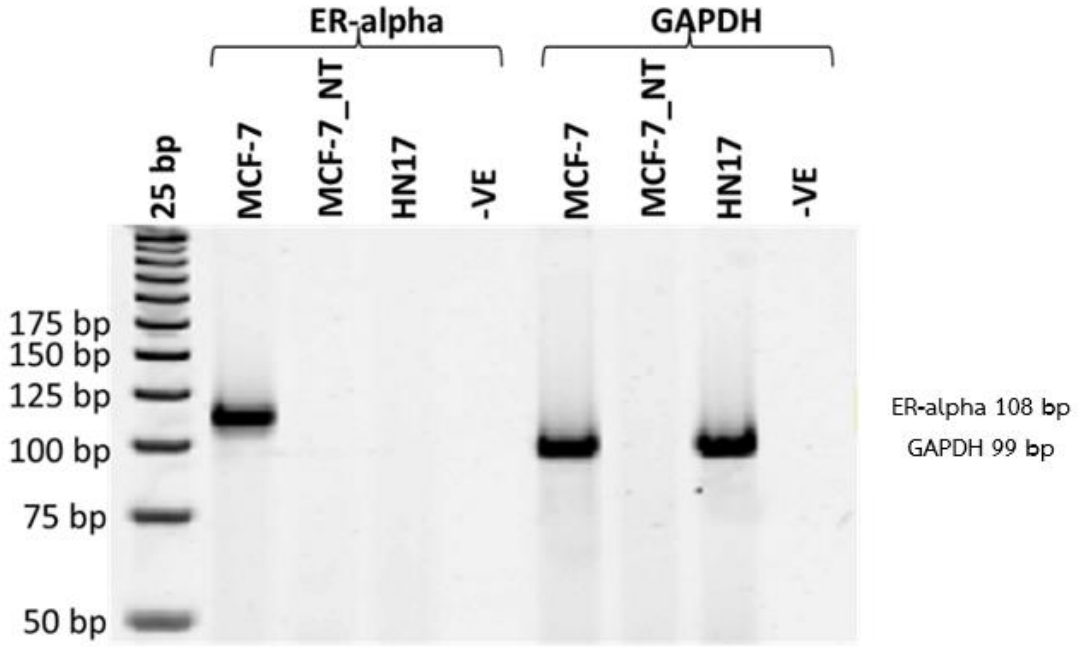


Figure 10. ER-alpha expression in MCF-7 cell lines. HN17 cell lines was used as negative control. *GAPDH* was used as positive control. MCF-7_NT represent non template cDNA

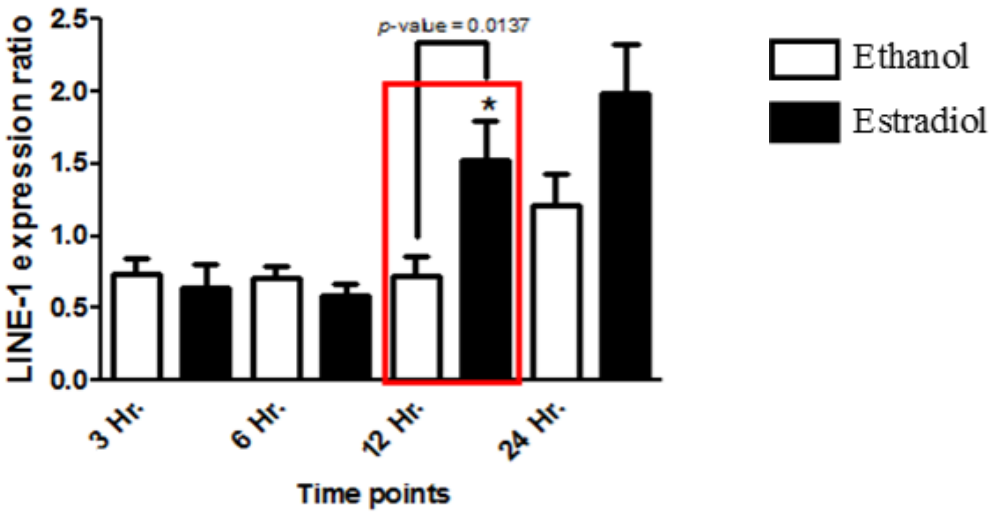


Figure 11. Time courses of E2 to intragenic LINE-1 expression in MCF-7 cells. Intragenic LINE-1s expression were induced by 100 nM E2 at various incubating time points (3, 6, 12 and 24 hours) in MCF-7 cell lines.

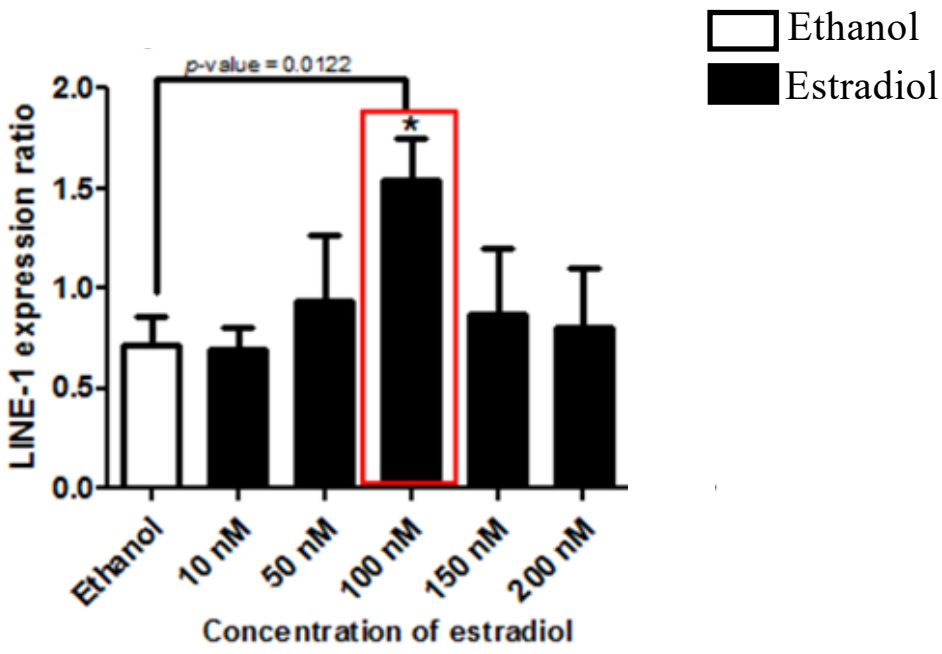


Figure 12. Dose response of E2 to intragenic LINE-1s expression in MCF-7 cells. Intragenic LINE-1s expression were induced by various concentrations for 12 hours in MCF-7 cell lines.

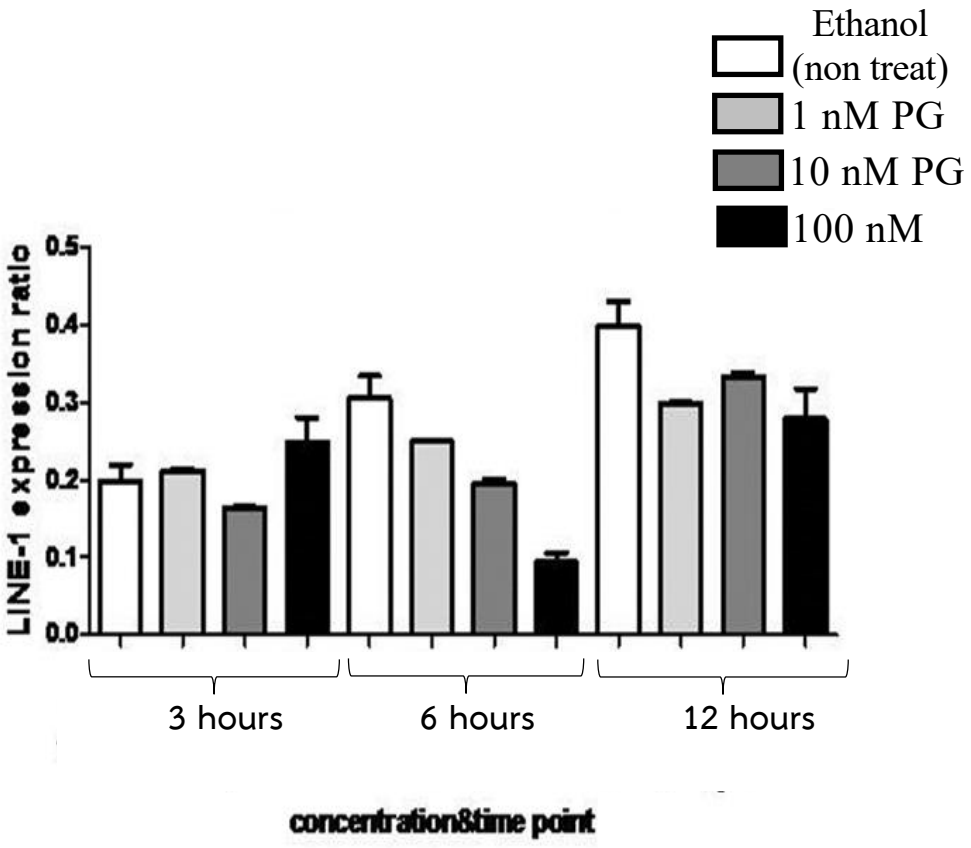


Figure 13. Time course and dose response of PG to intragenic LINE-1s expression in T47-D cells. Intragenic LINE-1s expression were induced by 1, 10 and 100 nM of PG for 3, 6 and 12 hours in T47-D cell lines.

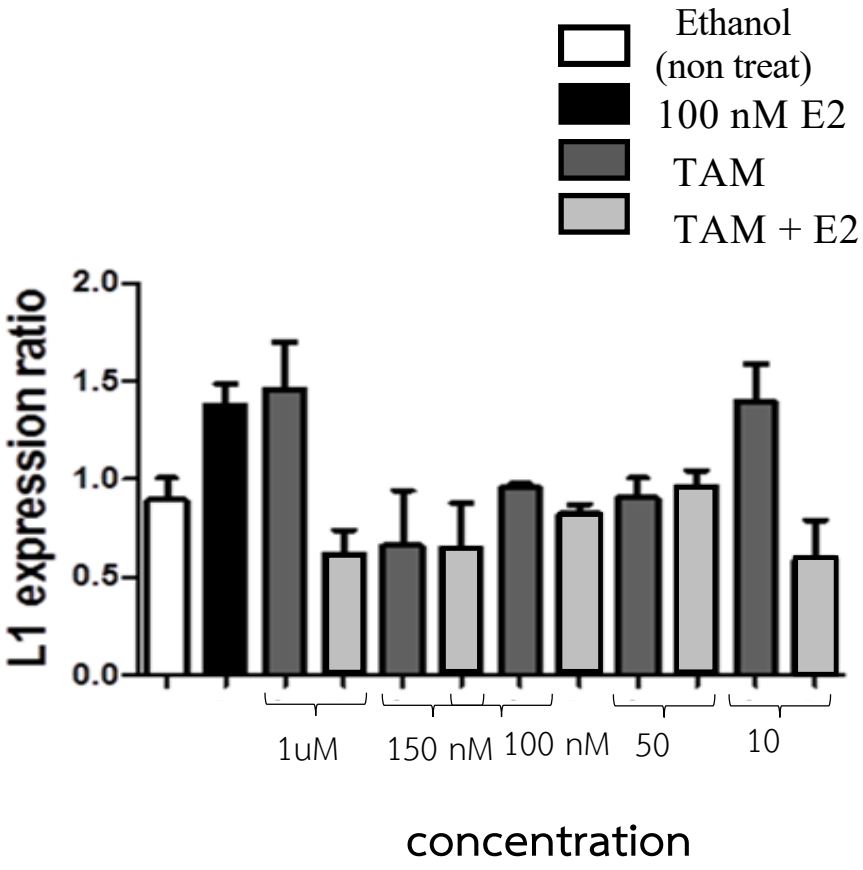


Figure 14. Dose response of TAM to intragenic LINE-1s expression in MCF-7 cells. The various concentrations of tamoxifen (TAM) at 1 uM, 150 nM, 100 nM, 50 nM, 10 nM were treated in MCF-7 cell lines for 12 hours.

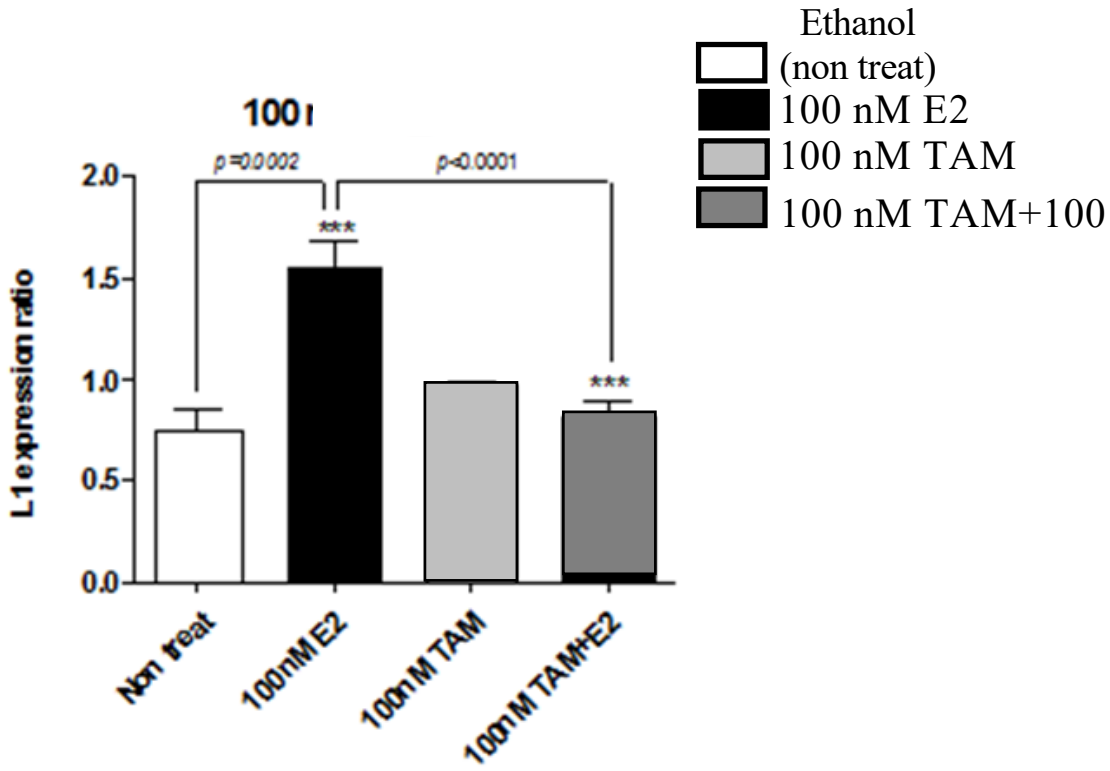


Figure 15. The effect of TAM to intragenic LINE-1s expression in MCF-7 cells. The expression of LINE-1s were induced by 100 nM E2, 100 nM tamoxifen (TAM), and 100 nM TAM with 100 nM E2 for 12 hours in MCF-7 cell lines.

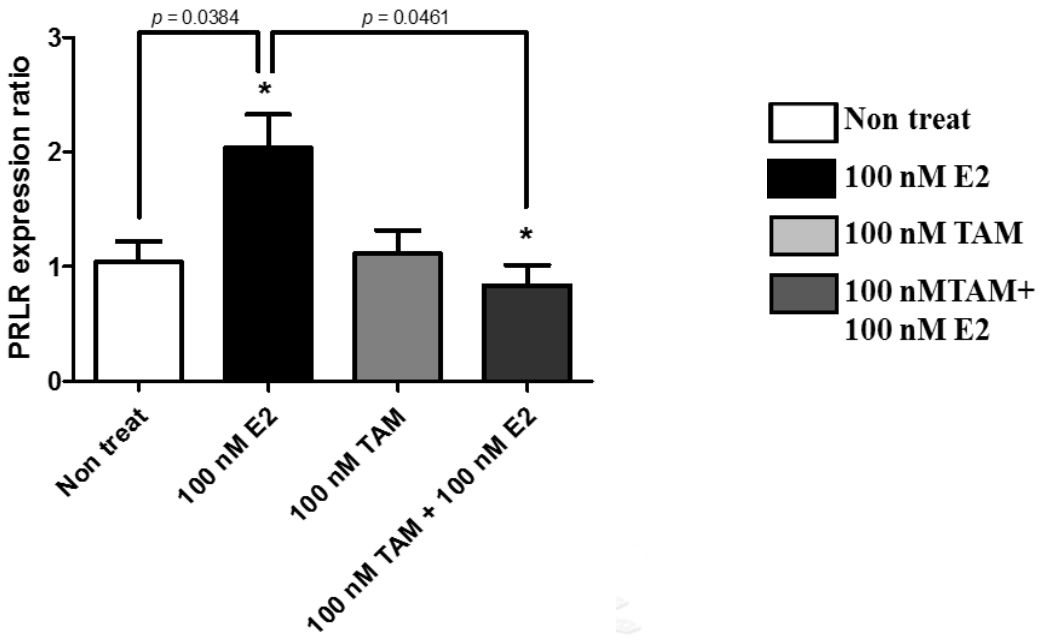


Figure 16. The effect of E2 and TAM to PRLR expression. The PRLR expression was induced by 100 nM E2, 100 nM tamoxifen (TAM), and 100 nM TAM with 100 nM E2 for 12 hours in MCF-7 cell lines.

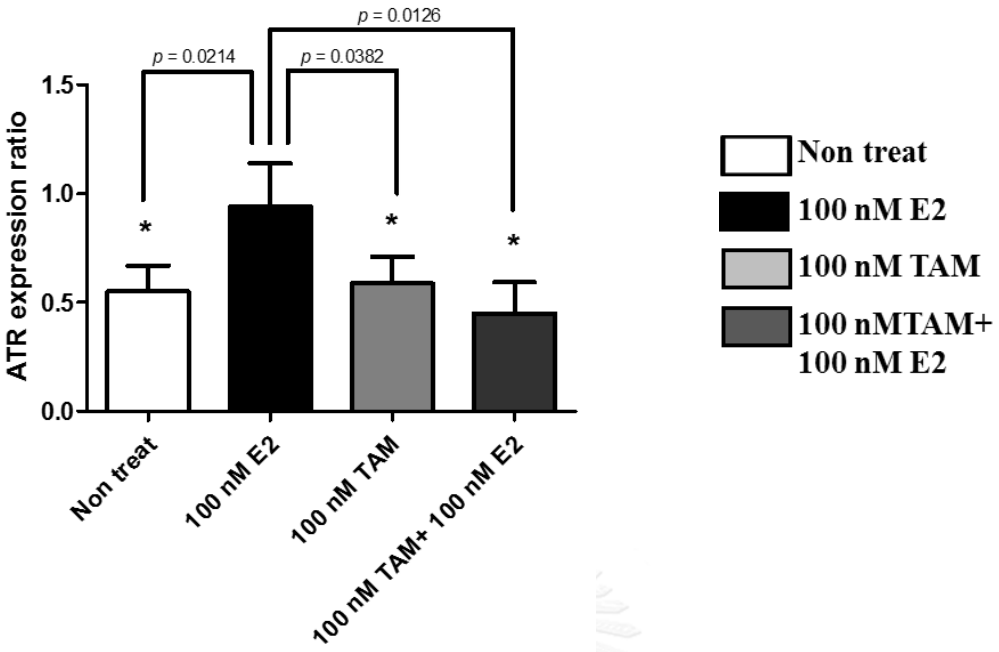


Figure 17. The effect of E2 and TAM to ATR expression. The ATR expression were induced by 100 nM E2, 100 nM tamoxifen (TAM), and 100 nM TAM with 100 nM E2 for 12 hours in MCF-7 cell lines.

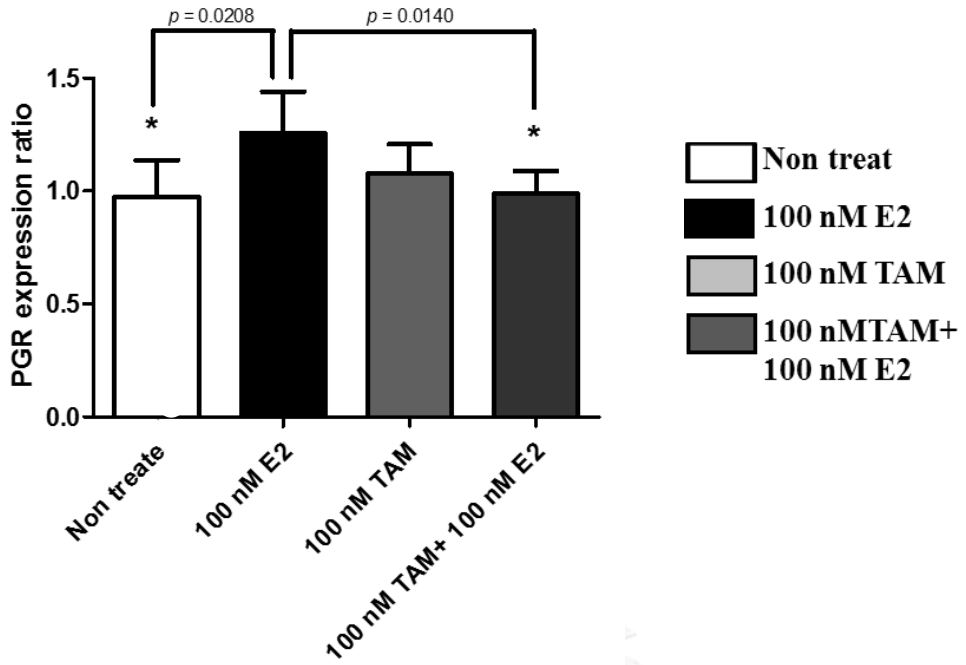


Figure 18. The effect of E2 and TAM to PGR expression. The PGR expression were induced by 100 nM E2, 100 nM tamoxifen (TAM), and 100 nM TAM with 100 nM E2 for 12 hours in MCF-7 cell lines.

4.2 The Effect of LINE-1 to candidate genes expression mediated through E2

The Unique RNA sequences of LINE-1s (combination of pre-mRNA of host gene and RNA of LINE-1s) can effect to gene expression. These sequences can transcribe from 5'UTR LINE-1 promoter which can transcribe in both forward (sense strand) and reverse (antisense strand) directions. So the effects of both strands to host genes expression were analyzed. Analyzing data were done by intersect sense or antisense LINE-1s to the up-regulated gene containing LINE-1 after treated with 100 nM E2 for 12 hours. From analyzing data showed that only antisense strand were related to up-regulated genes containing LINE-1 expression at 3 hours ((Table 2) p= 7.00E-04, 1.45 (1.17-1.81)). Meanwhile both sense and antisense strands of LINE-1s were found related to up-regulated genes containing LINE-1 expression at 6 hours, respectively (p=1.91E-03, 1.38(1.12-1.69); p=6.68E-05, 1.50(1.23-1.83) (Table 2). The results also similar to 12 hours after treated with 100 nM E2, both sense and antisense strands were found related to up-regulated gene containing LINE-1 expression (p=1.28E-05, 1.53(1.26-1.85); p=6.48E-07, 1.61(1.33-1.94) (Table 2).

Moreover, to validate the relation of LINE-1s to the up-regulated gene expression, number of up-regulate probes were divided into two groups. There were composed the group that up-regulated probes present before or after LINE-1 position. These up-regulated probe number were count and compared by Pearson's chi-squared test. Interesting results were shown that up-regulate probes number were found after LINE-1s more than in front of LINE-1s position. The similar results were shown in both sense and antisense strands of LINE-1 at 3 and 6 hours after treated with 100 nM E2, respectively (at 3 hours, p=5.67E-03, 3.2 (1.37-7.45); p= 4.60E-07, 4.47 (2.42-8.27); at 6 hours p= 7.15E-05, 4.7 (2.13-10.38); p= 4.52E-08, 4.4 (2.52-7.68) (Table 3). Percentage of up-regulated probe number in both sense and antisense LINE-1s at 3 and 6 hours were calculated. The higher percentage of up-regulated probes after LINE-1s position were found when compared to percentage of up-regulated probe numbers in front of LINE-1s position. These higher percentage of up-regulate probes number were found in both sense and antisense LINE-1 at 3 and 6 hours. For sense strands LINE-1s, percentage of before and after LINE-1s position at 3 hours were shown

as 23.08% and 48.96% meanwhile for antisense strands LINE-1s were shown as 20.83% and 54.06% (Figure 19). The similar results were shown at 6 hours in sense strands LINE-1s. Percentage of before and after LINE-1s position were shown as 36.84% and 73.27% meanwhile for antisense strands LINE-1s were shown as 67.84% and 32.39% (Figure 19).

From the percentage of up-regulated probe which were presented after LINE-1 position, the investigation of mRNA pattern (isoform) was done by RNA sequencing method. Genes containing LINE-1 with multiple isoforms were selected. Fragments per kilo base million (FPKM) of E2 treated samples were more than 0.1 and FPKM of non-treated samples were less than 0.1 at 12 hours were considered. Eleven genes were found in this condition which were composed ALPK1, CDKN2B-AS1, KIAA0586, MGST1, NAV2, NEK1, NREP, PHLDB2, RDX, RGS6 and RUNDC3B (Table 4). These genes were shown different increasing isoform in E2 treated samples more than in non-treated samples. The example of three candidate genes that present increasing isoform only in E2 treated samples such as Radixin (RDX), pleckstrin homology-like domain, family B, member 2 (PHLDB2) and microsomal glutathione transferase 1 (MGST1). The results were presented increasing of two isoforms in each genes (RDX: NM_001260494.1 and NM_001260496.1; PHLDB2: NM_001134437.1 and NM_001134438.1; MGST1: NM_001260511.1 and NM_001267598.1) (Figure 20). Interesting that these isoforms were presented after LINE-1 position.

Table 2. The effect of sense and antisense strands LINE-1s to up-regulated genes containing LINE-1s expression. Each strand of LINE-1s were analyzed in 100 nM E2 experiment in MCF-7 cells for 12 hours.

L1 strand			100 nM (up regulate genes)	
			up	not up
sense	P-value	3 hr.	5.48E-02	5.48E-02
	Odd Ratio		1.25	0.80
	Upper 95% CI		1.00	0.64
	Lower 95% CI		1.57	1.00
antisense	P-value		7.00E-04	7.00E-04
	Odd Ratio		1.45	0.69
	Upper 95% CI		1.17	0.55
	Lower 95% CI		1.81	0.86
sense	P-value	6 hr.	1.91E-03	1.91E-03
	Odd Ratio		1.38	0.73
	Upper 95% CI		1.12	0.59
	Lower 95% CI		1.69	0.89
antisense	P-value		6.88E-05	6.88E-05
	Odd Ratio		1.50	0.67
	Upper 95% CI		1.23	0.55
	Lower 95% CI		1.83	0.82
sense	P-value	12 hr.	1.28E-05	1.28E-05
	Odd Ratio		1.53	0.66
	Upper 95% CI		1.26	0.54
	Lower 95% CI		1.85	0.79
antisense	P-value		6.48E-07	6.48E-07
	Odd Ratio		1.61	0.62
	Upper 95% CI		1.33	0.51
	Lower 95% CI		1.94	0.75

Table 3. The effect of up-regulated probes numbers on sense and antisense strand LINE-1s to up-regulated genes containing LINE-1. Sense or antisense strands in up-regulated genes containing LINE-1 expression which were induced by 100 nM E2 for 3 and 6 hours were analyzed. The position of up-regulate probes in each strand of these genes were divided into TSS/L1 (before LINE-1s) or L1/AAA (after LINE-1s)

Probe				TSS/L1	L1/AAA
up regulate genes	sense L1	3 Hr.	P-value	5.67E-03	5.67E-03
			Odds ratio	0.31	3.2
			Lower 95% CI	0.13	1.37
			Upper 95% CI	0.73	7.45
	antisense L1		P-value	4.60E-07	4.60E-07
			Odds ratio	0.22	4.47
			Lower 95% CI	0.12	2.42
			Upper 95% CI	0.41	8.27
	sense L1	6 Hr.	P-value	7.15E-05	7.15E-05
			Odds ratio	0.21	4.7
			Lower 95% CI	0.1	2.13
			Upper 95% CI	0.47	10.38
	antisense L1		P-value	4.52E-08	4.52E-08
			Odds ratio	0.23	4.4
			Lower 95% CI	0.13	2.52
			Upper 95% CI	0.4	7.68

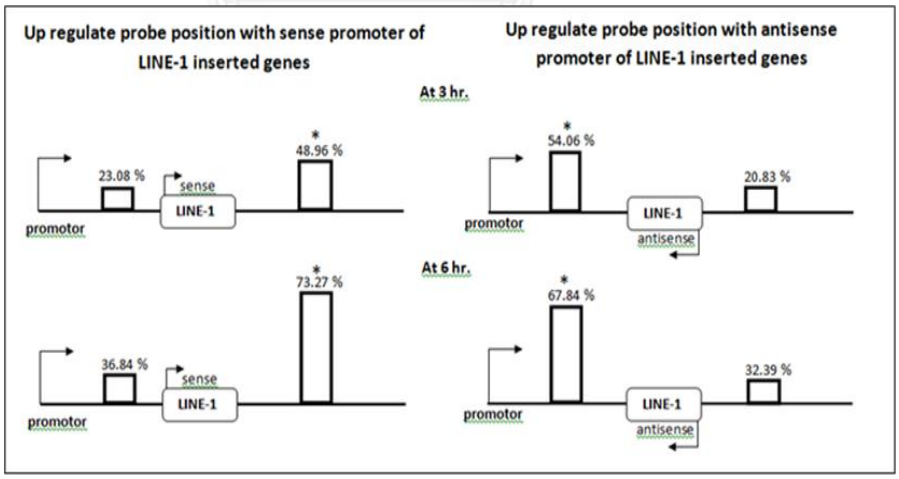
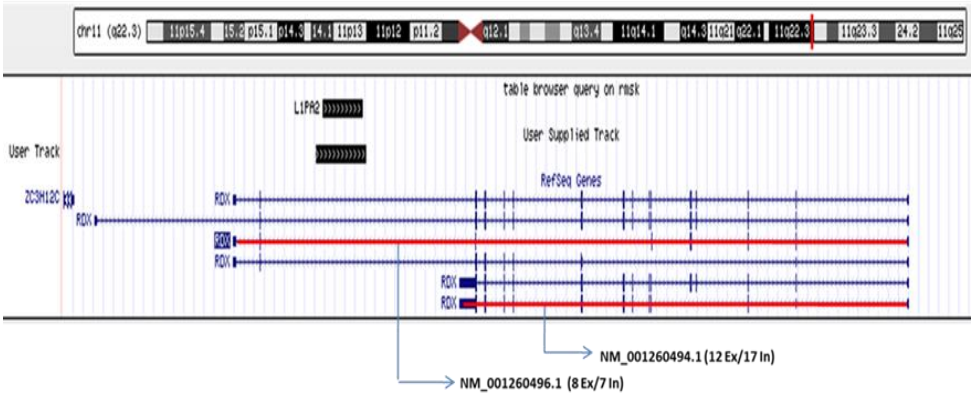


Figure 19. Percentage of up-regulated probes on LINE-1s position in up-regulated genes. The up-regulated probes were divided as before and after LINE-1s position for both LINE-1s sense and antisense strands. These percentage were analyzed in 100 nM E2 for 3 and 6 hours experiment.

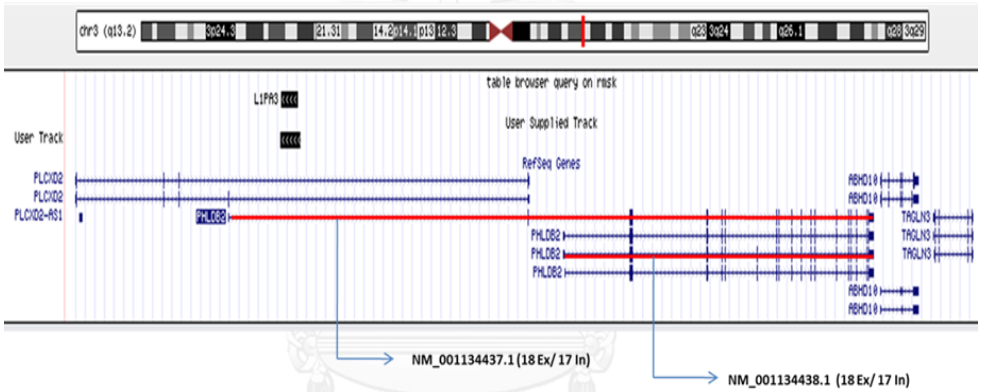
Table 4. Eleven genes containing LINE-1 were shown increasing isoform in E2 treated samples. Results from RNA sequencing technique, eleven genes containing LINE-1 were presented multiple isoforms. These isoforms were increased in 100 nM E2 sample (E) when compared to non treated samples (N). Guidelines for was the fragments per kilobase million (FPKM) in E2 treated samples (FPKM_E) were more than 0.1 and FPKM in non treated samples (FPKM_N) were less than 0.1.

Isoform	Gene	FPKM_N	FPKM_E	Log2FC
NM_001102406.1	ALPK1	0.000199	0.187958	9.8815
NM_001253884.1	ALPK1	0	0.269791	inf
NR_047542.1	CDKN2B-AS1	0	0.105033	inf
NR_047543.1	CDKN2B-AS1	0.000387	0.76561	10.9492
NM_001244190.1	KIAA0586	0	0.40706	inf
NM_001244191.1	KIAA0586	0.000834	0.126002	7.239
NM_001260511.1	MGST1	0	1.10319	inf
NM_001267598.1	MGST1	0.05876	0.115996	0.9812
NM_001244963.1	NAV2	0.084985	0.109569	0.3666
NM_145117.4	NAV2	0.000232	0.157051	9.4017
NM_001199398.1	NEK1	0.063425	0.173069	1.4482
NM_001199399.1	NEK1	0.013329	0.30757	4.5283
NM_001142474.1	NREP	0	0.124565	inf
NM_001142483.1	NREP	0	0.210755	inf
NM_001134437.1	PHLDB2	0	0.198882	inf
NM_001134438.1	PHLDB2	0.067361	0.705498	3.3887
NM_001260494.1	RDX	0	0.789005	inf
NM_001260496.1	RDX	0.09243	0.337069	1.8666
NM_001204418.1	RGS6	0	0.727508	inf
NM_001204422.1	RGS6	0	0.43009	inf
NM_001204423.1	RGS6	0.083377	0.281469	1.7553
NM_001134406.1	RUNDC3B	0.000166	0.249207	10.5486
NM_138290.2	RUNDC3B	0	0.159001	inf

RDX



PHLDB2



MGST1

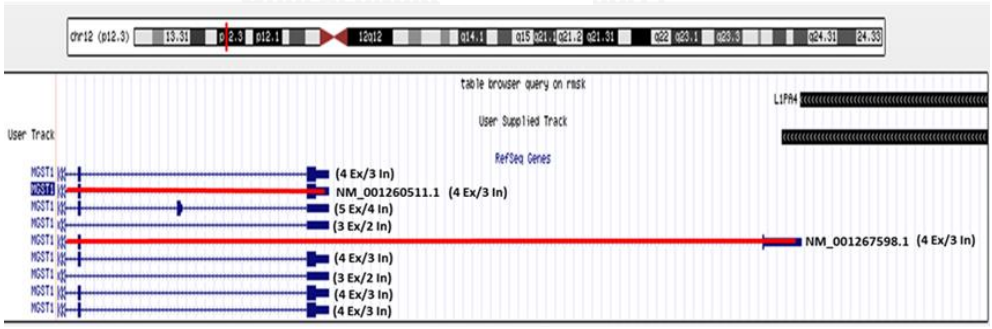


Figure 20. Three candidate genes were shown Increasing of some isoforms in E2 treated sample. RDX, PHLDB2 and MGST1 in 100 nM E2 treated samples were present isoform after LINE-1 position (RDX: NM_001260496.1 and NM_001260494.1; PHLDB2: NM_001134437.1 and NM_001134438.1; MGST1: NM_001260511.1 and NM_001267598.1).

4.3 The interaction between E2 and genes containing LINE-1 regulation

4.3.1 The important of EREs in the interaction of ER complex to regulate gene containing LINE-1 expression

The genomic pathway which were driven from E2 relate to ERE was studied. To validate the important of ERE in up-regulated gene containing LINE-1 mechanism, genes in this group were divided into 2 groups. There were genes containing LINE-1 with or without ERE. The intersection of genes containing LINE-1 with or without ERE to up-regulated gene which induced by 100 nM E2 for 3, 6 and 12 hour were done. From analyzed the expression data, there is no different result between both groups even in different incubating times. The significant results in up-regulated genes containing LINE-1 with ERE for 3, 6 and 12 hours were included p= 6.38E-07, 1.43(1.24-1.64); p= 4.50E-13, 1.59(1.40-1.81); p= 1.18E-15, 1.64(1.45-1.85), respectively (Table 5). Meanwhile the result in up-regulated genes containing LINE-1 without ERE for 3, 6 and 12 hours were included p= 3.30E-07, 1.51(1.29-1.77); P= 7.01E-13, 1.68(1.46-1.94); p= 3.78E-13, 1.66(1.45-1.51), respectively (Table 5). So, that mean ERE may not directly related in E2 induces genes containing LINE-1 mechanism through LINE-1s.

4.3.2 The relation of E2 early immediate genes to ER complex to regulate genes containing LINE-1 expression

The identification of ER binding sites which involved in up-regulated genes containing LINE-1 were analyzed. Ten types of E2 early immediate genes were intersected with up-regulated genes containing LINE-1 which were regulated by 100 nM E2 for 12 hours. They were composed c-jun#1, c-jun#2, c-fos#1, c-fos#2, E2F1, cathepsinD, forkhead, Ap1, C/EBP and Otc. The results were shown significant of c-fos#2 (p= 1.81-05, 1.39(1.20-1.62)) and C/EBP p= 8.69E-06, 1.42(1.22-1.66) at 6 hours meanwhile the significant of c-fos#2 (p= 6.90E-09, 1.50(1.31-1.73), forkhead (p= 2.95E-03, 2.16(1.36-5.21) and C/EBP (9.82E-08, 1.47(1.28-1.7)) were found at 12 hours ($p< 0.05$) (Table 6). Moreover, the significant were increased to $p<0.005$, c-fos#2 and C/EBP at 6 and 12 hours still present the significant results ((at 6 hours c-fos#2 (p= 1.41E-02,

1.24(1.04-1.47)); C/EBP (p= 3.69E-03, 1.29(1.09-1.53)) at 12 hours c-fos#2 (p= 1.06E-05, 1.40(1.26-1.63)); C/EBP (p= 7.46E-05, 1.37(1.17-1.60)) (Table 7).

4.3.3 The identification of related E2 early immediate genes binding sites on 5'LINE-1s promotor.

The expression of c-fos#2 and C/EBP at various incubating time point (4, 6, 8 and 12 hours) with 100 nM E2 were done by real time RT PCR. The expression of both c-fos#2 and C/EBP were compared between with or without 100 nM E2 at different incubating times. High expression of c-fos (p=0.388) and C/EBP were presented at 6 hours after treated with 100 nM E2 when compared to non-treated (Figure 21 and 22). Moreover, to prove function of c-fos and C/EBP in the relationship to E2 and intragenic LINE-1s, their binding site at 5'LINE-1 promotor in candidate genes were proved by ChIP. The E2 incubating times were composed 0, 8, 10 and 12 hours. The specific antibody (c-fos) were not shown at 0, 8 and 10 hours, but only IgG were shown in E2 treated samples. At 12 hours, binding site of c-fos to 5'LINE-1 promoter in both PRLR and PGR containing LINE-1 binging site were shown only in E2 treated samples but not shown in non-treated samples (Figure 23). However, the different binding times of C/EBP to 5'LINE-1 promoters were presented in PRLR containing LINE-1 binging site. The non specific binding of C/EBP were shown in both treated and non treated samples at 8, 10 and 12 hours (Figure 24).

Table 5. The expression of up-regulated genes containing LINE-1 with ERE or without ERE. The expression of up-regulated genes containing LINE-1 expression which were induced by 100 nM E2 for 3, 6 and 12 hours.

Experiments		100 nM (up regulate genes)	
		L1 with ERE	L1 without ERE
P-value	3 hr.	6.38E-07	3.30E-07
Odd Ratio		1.43	1.51
Upper 95% CI		1.24	1.29
Lower 95% CI		1.64	1.77
P-value	6 hr.	4.50E-13	7.01E-13
Odd Ratio		1.59	1.68
Upper 95% CI		1.40	1.46
Lower 95% CI		1.81	1.94
P-value	12 hr.	1.18E-15	3.78E-13
Odd Ratio		1.64	1.66
Upper 95% CI		1.45	1.45
Lower 95% CI		1.85	1.91

Table 6. Up-regulated gene containing LINE-1 with E2 early immediate genes binding sites. E2 early immediate genes expression which induced by 100 nM E2 for 3, 6 and 12 hours in MCF-7 cell lines ($p<0.05$).

Expression	Experiments	c-jun#1	c-jun#2	c-fos#1	c-fos#2	E2F1	cathepsinD	Forkhead	AP1	C/EBP	Oct
P-value	1 (3H)	7.67E-01	8.13E-01	8.13E-01	4.79E-01	2.87E-01	7.63E-01	5.31E-01	9.67E-01	2.41E-01	5.38E-01
Odd Ratio		0.74	0.96	0.96	1.08	0.73	0.93	1.39	0.99	1.13	1.22
Upper 95% CI		0.10	0.71	0.71	0.88	0.41	0.58	0.49	0.69	0.92	0.65
Lower 95% CI		5.57	1.32	1.32	1.33	1.31	1.49	3.92	1.44	1.4	2.27
P-value	2 (6H)	8.39E-01	8.41E-01	8.41E-01	**1.81E-05**	4.97E-01	1.97E-01	6.04E-02	6.82E-01	**8.69E-06**	9.70E-01
Odd Ratio		0.86	1.02	1.02	**1.39**	1.14	1.24	2	0.94	**1.42**	1.01
Upper 95% CI		0.20	0.81	0.81	**1.20**	0.78	0.89	0.95	0.7	**1.22**	0.6
Lower 95% CI		3.76	1.30	1.30	**1.62**	1.68	1.74	4.23	1.26	**1.66**	1.71
P-value	3 (12H)	6.07E-01	7.81E-02	7.81E-02	**6.90E-09**	3.19E-01	1.50E-02	**2.95E-03**	5.40E-01	**9.82E-08**	8.66E-01
Odd Ratio		0.68	1.21	1.21	**1.50**	1.19	1.44	**2.16**	1.08	**1.47**	1.04
Upper 95% CI		0.16	0.98	0.98	**1.31**	0.84	1.07	**1.36**	0.84	**1.28**	0.64
Lower 95% CI		2.98	1.49	1.49	**1.73**	1.70	1.94	**5.21**	1.41	**1.7**	1.69

Table 7. Up-regulated gene containing LINE-1 with c-fos and C/EBP binding sites. Up-regulated E2 early immediate genes (c-fos#2 and C/EBP) expression which induced by 100 nM E2 for 3, 6 and 12 hours in MCF-7 cell lines (p=0.005)

		c-fos#2	C/EBP
Expression	**Experiments**	**0.005**	**0.005**
P-value	**1 (3H)**	6.11E-01	4.77E-01
Odd Ratio		1.07	1.10
Upper 95% CI		0.83	0.85
Lower 95% CI		1.37	1.41
P-value	**2 (6H)**	**1.41E-02**	**3.69E-03**
Odd Ratio		**1.24**	**1.29**
Upper 95% CI		**1.04**	**1.09**
Lower 95% CI		**1.47**	**1.53**
P-value	**3 (12H)**	**1.06E-05**	**7.46E-05**
Odd Ratio		**1.40**	**1.37**
Upper 95% CI		**1.21**	**1.17**
Lower 95% CI		**1.63**	**1.60**

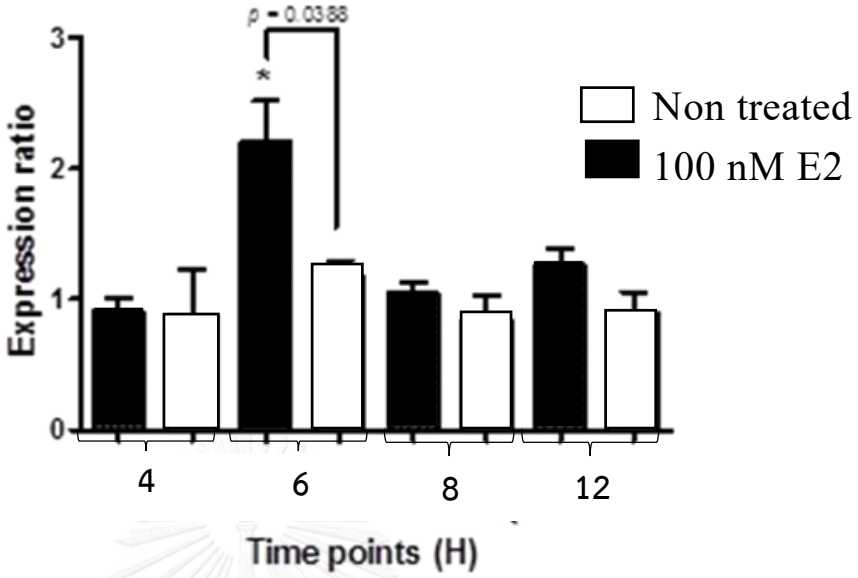


Figure 21. Time courses of c-fos expression ratio after induced by E2. c-fos expression was induced by 100 nM E2 for 4, 6, 8 and 12 hours compared to non treated.

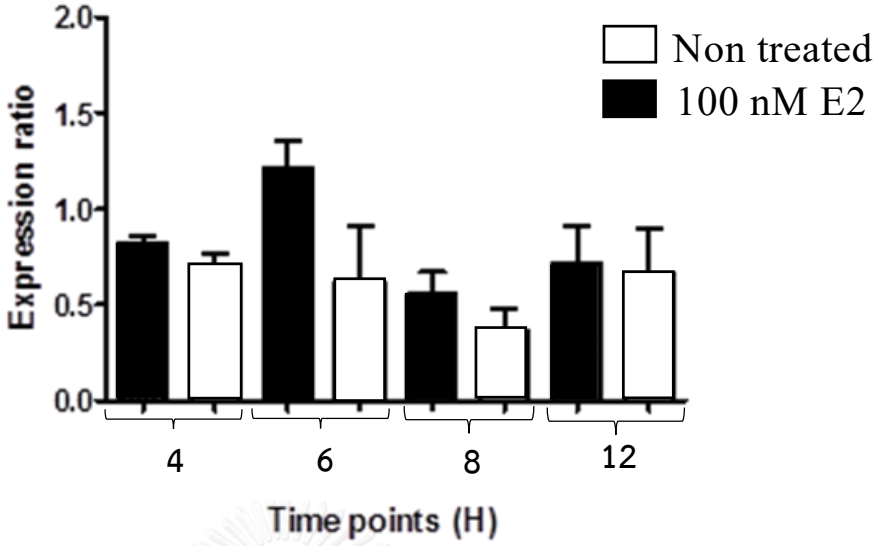


Figure 22. Time courses of C/EBP expression ratio after induced by E2. C/EBP expression was induced by 100 nM E2 for 4, 6, 8 and 12 hours compared to non treated.

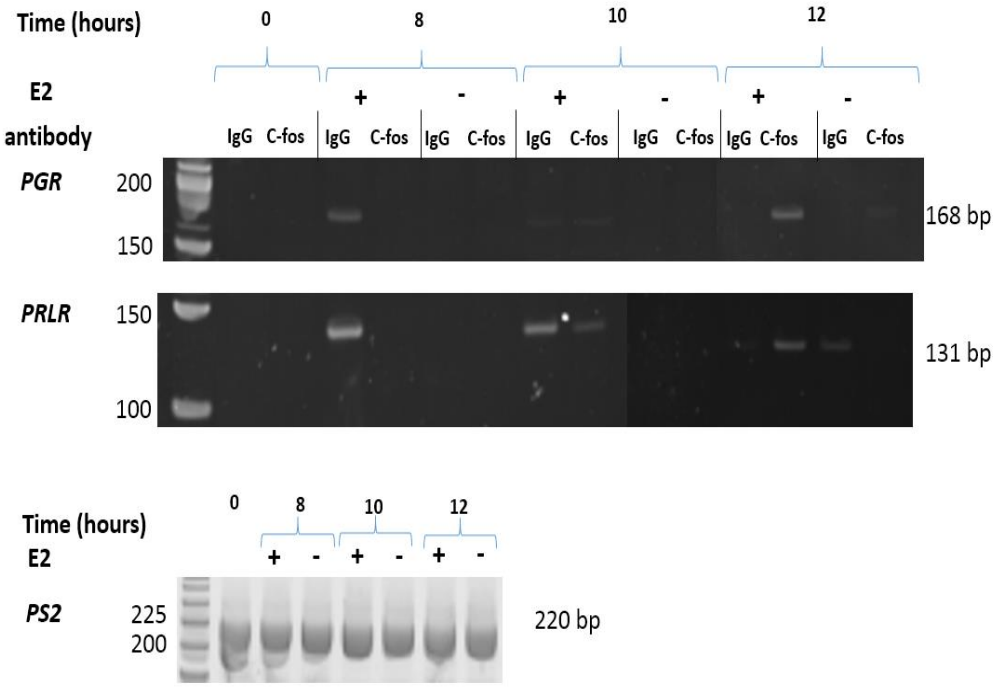
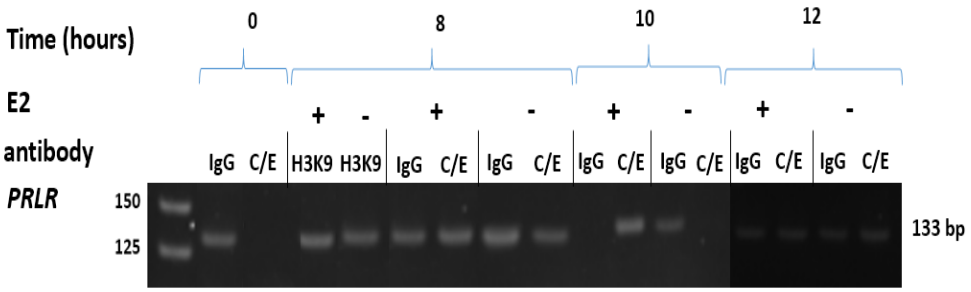


Figure 23. Binding site of c-fos on 5' LINE-1 promotor by ChIP assay. The 100 nM E2 with incubating time at 12 hours were presented sharp product size of c-fos more than IgG in non treated samples. Input DNA were proved by PS2 (220 bp). IgG: immunoglobulin G antibody (negative control); c-fos: c-fos antibody. The c-fos products were investigated at 0, 8 10 and 12 hours (PGR:168 bp and PRLR: 131 bp).

Figure 24. Binding site of C/EBP on 5' LINE-1 promotor by ChIP assay. Non specific products were found after treated with 100 nM E2 with incubating time at 12 hours (PRLR: 131 bp).



Chapter 5 Discussion

Sex steroid hormones are the important regulator to drive gene expression in various pathways. From the expression array analysis, genes containing intragenic LINE-1 were related in up-regulated mechanism which were induced by 100 nM E2 especially at 12 hours. Meanwhile, down-regulated genes containing LINE-1 were related to 10 nM PG at 16 hours in breast cancer cells. Moreover, both up and down-regulated genes were found relate to 10 nM DHT at 16 hours in breast cancer cells. These preliminary result suggesting that intragenic LINE-1 might play a role in host gene regulation which were induced by sex steroid hormones.

From previous report, unique LINE-1s RNA sequences were present in both forward and reverse direction. They were produced from the active 5'LINE-1 bidirectional promotors. These sequences are the combination of pre-mRNA of host gene and RNA of LINE-1 (Matlik et al., 2006; Weber et al., 2010). In forward direction of LINE-1s unique RNA sequences, the transcriptions can produce from LINE-1 position and continue to the end of the host genes (Kitkumthorn and Mutirangura, 2011). Producing of these transcribes might be effect to increase mRNA and cause up-regulated genes containing LINE-1. So, suggesting that forward direction of unique LINE-1 RNA sequences might be the important mechanism in up-regulate genes containing LINE-1 which induced by 100 nM E2 or 10 nM DHT for 12 hours and 16 hours, respectively. In PG experiment, non stable of LINE-1s expression results in vitro experiment were presented suggested that other mechanism may relate with PG to down-regulated genes containing LINE-1. For the second type of LINE-1 RNA, reverse direction of unique RNA sequences may support the down-regulation mechanism in host genes. These sequences can regulate RNA-induced silencing complex (RISC) and AGO2 protein which can destroy mRNA of host genes and repress host gene expression (Aporntewan et al., 2011). This suggestion may also explain in DHT to down-regulated genes containing LINE-1 expression.

Here, E2 experiment was done and supported the relationship of 100 nM E2 in up-regulated genes containing LINE-1 at 12 hours. The high concentration of E2 (100 nM) treatment was found greatly affect in proliferation, migration and invasion of breast cells (Zhao et al., 2014). At the higher concentration (150 and 200 nM), decreasing of LINE-1 expressions were found. In previously published study suggests that ER protein in uterus may be ubiquitinated. ER protein exhibits a short half-life in estrogen target tissues which is rapidly degraded in mammalian cells. The half-life of ER with E2 is about 3-4 hours meanwhile in the absence of E2 is about 5 days. It is possible that ER would be a target of the ubiquitin–proteasome degradation pathway (Nirmala and Thampan, 1995; Pakdel et al., 1993). Moreover, hormone-induced ER degradation was found and served to control physiological responses in estrogen target tissues by down-regulating ER, which limit the expression of estrogen-responsive genes estradiol (Nawaz et al., 1999). Then, the influence of Tamoxifen was shown the opposite results in down-regulated intragenic LINE-1 and down-regulated genes containing LINE-1 expression. Tamoxifen can bind to ER in the cancer cells and block estrogen from attaching to their receptors. The ER/Tamoxifen complex recruit co-repressors and binds to DNA, so the effect of E2 were slowed or stopped (Wang et al., 2004; Shang et al., 2000).

Moreover, the increasing of up-regulated probes and some isoform in up-regulated gene containing LINE-1, which were presented after LINE-1 position, were supported the important function of forward unique LINE-1 RNA sequences. More supported results were presented by the significant of LINE-1s sense strands (forward direction) in up-regulated genes containing LINE-1 from array expression analysis. Antisense strands of LINE-1 (reverse direction) were also found, but more function still need to explain for the relation to up-regulated genes containing LINE-1. From RNA sequencing results, increasing of some isoforms after LINE-1 position in E2 treated cells were shown in up-regulated genes containing LINE-1. These showed clearly understand about the influence of E2 to up-regulated genes with intragenic LINE-1. The increasing of isoforms in each genes after LINE-1 position also increase the chance to detect up-regulate probes in array expression. So, up-regulated genes were identified when compared to control. The example of three genes (RDX, PHLDB2 and MGST1) were

found in this pattern. Interesting that function of them have been reported to link with cancers progression. First, the expression of RDX which is the one of cell structure-related proteins (ERM family) was found related in cells proliferation in cancer pathogenesis (Herman et al., 2010). Second, the results from human protein atlas stained tissues was shown high expression protein from PHLDB2 in breast cancer tissue (Mosca et al., 2010). The last gene is MGST1. This gene has been report that up-regulated in various malignant tissues when compared to normal tissues (Morgenster et al., 2011).

For interaction between E2 and gene containing LINE-1 regulation part, EREs were not shown the relation in up-regulated genes containing LINE-1 mechanism. So, the second mode of action, DNA-binding transcription factor to mediate ER association with DNA were studied. This mechanism is generally known as “transcriptional cross-talk” (Gottlicher et al., 1998; O'Lone et al., 2004). From previous reported, the result was shown that 35% of primary E2-responsive genes were related to transcription via ER-indirect DNA association (O'Lone et al., 2004). In this study, results from array expressions were shown the relation of two E2-responsive genes (c-fos and C/EBP) in up-regulated gene containing LINE-1 mechanism. Their expression were proved in vitro and were shown increasing at the same period (6 hours) after incubated with 100 nM E2. Suggesting that both c-fos and C/EBP may act as necessary transcription factors which were activated by 100 nm E2 in this mechanism.

The proto-oncogene c-fos encodes a nuclear protein and interact with c-jun to form the heterodimeric activating protein-1 (AP-1) transcription factor complex. (Curran et al., 1998). c-fos is an immediate-early gene which is induced in cells or tissues by various activator such as E2 (Rivera and Greenberg, 1990). Moreover, c-fos was found as a critical AP-1 protein involved in regulating MCF-7 cells proliferation which were found in cancers (Preston et al., 1996; Lu et al., 2005). Meanwhile C/EBP has been shown to interact with estrogen receptor binding sites, but not to the negative controls in MCF-7 cells and act as adjacent binding sites for cooperating factors (Carroll et al., 2006). Moreover, C/EBP also found the direct involvement of a transcription factor in the mechanism of cyclin D1 (CCND1) action (Lamb et al. 2003). CCND1 has been reported about the overexpressed in human tumor cells (Hall and Peters, 1996).

Previous studies suggest that AP-1 not only plays a critical role in the breast cell growth and transformation but also regulates the expression of cyclin D. These relative function are also leading to cell cycle progression and breast cancer cell proliferation (Shen et al., 2007). From the relationship of both c-fos and C/EBP to promote cancer, these were supported their activation in up-regulated genes. Their binding site which were proved from ChIP method, were confirmed the closely relate function to increasing gene expression. Interesting that the binding site of both c-fos and C/EBP were found on 5'LINE-1s even in the different binding periods (12 and 10 hours respectively). These were proved the important of intragenic LINE-1s for increasing gene expression and promote breast cancer. The binding period of c-fos to 5'LINE1 promotor was found later than C/EBP. Suggesting c-fos may needs more factors to interact before binding at 5'LINE-1 promotor. However, non specific of binding site which were found in C/EBP on 5' LINE-1 in MCF-7 cells still need more knowledge to fulfill these mechanisms.

Chapter 6 Conclusion

The regulation of genes containing LINE-1 expression by sex steroid hormones were discovered. The expression array showed that genes containing LINE-1 were up-regulated by treating with 100 nM E2 at 3, 6, and 12 hours in MCF-7 cells. In contrast, down-regulated genes containing LINE-1 were found in PG treated with 10 nM in T47-D for 16 hours. Moreover, 10 nM DHT treated in ZR-75-1 cells for 16 hours were shown the relation in both up and down-regulated genes containing LINE-1.

In vitro studied was also supported the results from expression arrays in E2 experiment. The effect of 100 nM E2, which were incubated for 12 hours in MCF-7 cells, can induce intragenic LINE-1s expression in up-regulated genes containing LINE-1. Interesting that, these up-regulated genes were shown the new pattern of isoform which were present after intragenic LINE-1s position only in 100 nM E2 treated samples. Transcriptional cross talk between E2 and two E2 early immediate genes were discovered. These two transcription factors were included c-fos and C/EBP which were presented high expression at 6 hours after treating with 100 nM E2. Their binding sites were found at 5'LINE-1 promotor, but different binding times of them were presented. Binding time at the binding site of c-fos was 12 hours meanwhile C/EBP was 10 hours after treated with E2. The longer binding time of c-fos may need more another initiator for supporting in the co-operation before activate LINE-1 promotor. Meanwhile, the function of C/EBP which present the non-specific binding times still not clear. From all results were shown that 100 nM E2 can act as initiators to activate intragenic LINE-1s transcription. ER complexes may induced the binding of transcription factors (c-fos) to attach at their binding site at 5'LINE-1 promotor. These mechanism can cause the activation of LINE-1 promotor, especially in sense strands, and then can up-regulated genes by new isoforms production (short form) (Figure 25).

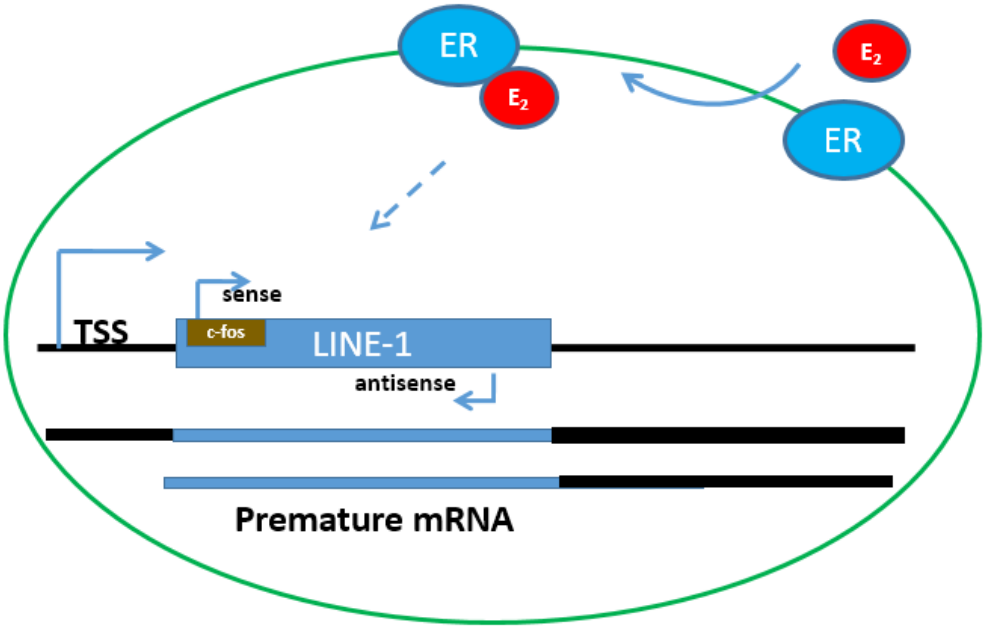


Figure 25. E2 regulated genes containing LINE-1 expression model.

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APPENDIX

Appendix I

	Microarray experiment
1) Up- or down regulated genes with LINE-1	2) Not up- or down regulated genes with LINE-1
3) Up- or down regulated genes without LINE-1	4) Not up- or down regulated genes without LINE-1

The correlation between the expression of genes from microarray libraries and genes containing or without LINE-1. The microarray data were intersected with the gene in 4 groups as 1) genes containing LINE-1 and regulated in array experiments 2) genes containing LINE-1 and not regulated in the arrays experiment 3) genes without LINE-1 and regulated in the experiment 4) genes without LINE-1 and not regulated in the array experiments. The 2x2 table was provided for frequency of occurrences. The odds ratio of up- or down- regulated in genes containing LINE-1 was calculated by a dividing the probability of an event happening by the probability of the event not happening. The odds ratio of gene containing LINE-1 in up or down-regulated that related to the experiment was analyzed. The number of up- or down- regulated genes containing LINE-1 was divided by the number of not up- or not down- regulated genes containing LINE-1. Then, the number of in up- or down-regulated gene not containing LINE-1 was divided by the number of not up-or not down-regulated genes that did not containing LINE-1. The odd ratio of more than 1.0 indicated the association between effect of sex steroid hormones and genes containing LINE-1 expression.

Appendix II

1. Reagents

DMEM (GIBCO®, USA)

Fetal Bovine Serum (Bio Whittaker®, Maryland, USA)

Charcoal stripped Fetal Bovine Serum (Sigma®, UK)

DMEM/High glucose, with L-glutamine; without phenol red, sodium pyruvate (HyCloneTM, Logan, Utah)

Antibiotic-Antimycotic (100X) (Invitrogen®, U.S.A.)

DMSO (Sigma®, UK)

Ficoll-HypaqueIsoprep (Amersham Pharmacia®, Uppsala, Sweden)

Phosphate Buffer Saline (Sigma®, UK)

Trypan Blue (Sigma®, UK)

17β estradiol (Sigma®, UK)

4-HYDROXY-, (Z)- (100 nM; Merck®, Germany).

Absolute ethanol (Merck®, Germany)

Trizol reagent (Life Technologies, Inc.)

RNase-free DNaseI (Fermentas)

Normal mouse IgG antibody (Santa Cruz Biotechnology, UK).

c-fos antibody (Cell signaling technology, USA)

C/EBP antibody (Cell signaling technology, USA)

2. Materials

6-wells flat plate (Costar®, USA)

Automatic pipette (Gilson®, France)

Conical tube 15 and 50 ml (Falcon®, USA)

Counting chamber (Celeromics®, France)

Cryotube (Sarstedt®, Germany)

Disposal Serological pipette 2, 5, 10 and 25 ml (Costar®, USA)

Flask 50 and 250 cm3 (Nunc®, Denmark)

Glove

Micro centrifuge tube (Eppendorf®, USA)

Pipette boy and pipette tip

3. Instruments

RNA sequencing MiSeq

Centrifuge

Biological Safety Cabinet type II

CO2 incubator (Forma Scientific®, USA)

Freezer -80oC

PCR machine (Thermo Scientific®, USA)

Real time RT PCR (Bio-Rad Laboratories®, USA)

Sonicater (Qsonica®, USA)

Rotater (Qsonica®, USA)

Refrigerator

Water bath (Shel lab, USA)

4. Programs

CU-DREAM Ex (http://pioneer.netserv.chula.ac.th/~achatcha/CU-DREAM/)

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/)

Genecard® (http://www.genecards.org/, Lifemap Science, USA)

Oligoanalyzer 3.1 (http://sg.idtdna.com/calc/analyzer)

RSAT (Regulatory Sequence Analysis Tools) (http://www.rsat.eu)

VITA

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