

HORMONAL REGULATION OF CARBOXYPEPTIDASE-D GENE

TRANSCRIPTION IN BREAST CANCER CELLS

by

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ABSTRACT

Carboxypeptidase-D (CPD), bound to the plasma membrane, cleaves C-terminal arginine from extracellular substrates. Arginine is converted to nitric oxide (NO), which can promote tumour progression. We have previously reported that 17 β -estradiol (E2) and prolactin (PRL) upregulate CPD mRNA/protein levels to increase NO production for the survival of human breast cancer cells. Androgen also upregulates CPD expression to increase NO production and survival of prostate cancer cells. The human CPD gene promoter contains a consensus γ -interferon-activated sequence (GAS) and several putative androgen response elements (AREs) that could potentially bind PRL-activated transcription factor Stat5 and the ligand-bound androgen receptor (AR), respectively. This study investigated regulation of the CPD gene by E2, PRL, and synthetic androgen R1881, in human MCF-7 and T47D breast cancer cell lines. CPD mRNA and protein levels were elevated by E2, PRL, and R1881, in a time- and dose-dependent manner. Upregulation of CPD mRNA by PRL and R1881 was abolished by actinomycin-D, suggesting transcriptional regulation by these two hormones. E2 acts by increasing CPD mRNA stability. The 2.0-kbp CPD gene promoter construct, containing a consensus GAS and three putative AREs, was stimulated by PRL and R1881, but not E2. PRL- and R1881-stimulated CPD promoter activities were not affected by deletion of ARE-2 and ARE-3, suggesting that the GAS, and in particular, ARE1, are active hormone response elements. PRL-stimulated Δ GAS-CPD promoter activity was abolished by the mutation of GAS (Δ GAS-CPD, ARE-1 intact). Surprisingly, R1881 was unable to stimulate the same promoter. However, Δ GAS-CPD promoter activity was restored when PRL and R1881 were administered together, and further enhanced by ectopic transfection of Stat5, suggesting cooperativity between Stat5 and the AR. Furthermore, ChIP analysis confirmed that PRL-activated Stat5 and the liganded AR bound to GAS and ARE-1, respectively. In summary, PRL and R1881, acting through Stat5 and AR act in concert to stimulate CPD gene transcription and expression. E2 stabilizes the CPD mRNA to maintain CPD mRNA/protein levels. Taken together, our results implicate the cooperative action of the AR and PRL receptor signalling pathways in breast cancer.

LIST OF ABBREVIATION AND SYMBOLS USED

APS	ammonium persulphate
AR	androgen receptor
ARE	androgen response element
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer type 2 susceptibility protein
cGMP	cyclic GMP
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovary
CIS	cytokine-inducible SH2-containing protein
CKB	creatine kinase B
CP	carboxypeptidase
CPD	Carboxypeptidase-D
CPD-N	nuclear-targeted CPD isoform
CRH	cytokine receptor homology domain
CSSM	charcoal-stripped serum containing medium
DBD	DNA-binding domain
DHT	dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
E2	17 β -estradiol
ECD	extracellular domain
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
ERE	estrogen response elements
FBS	fetal bovine serum
GAS	γ -interferon activated sequence
GH	growth hormone

GnRH	gonadotropin-releasing hormone
Her2/neu	epidermal growth factor receptor 2
hPRL/hPRLR	human prolactin/prolactin receptor
HSP	heat-shock protein
ICAM1	Intercellular Adhesion Molecule 1
IgG	immunoglobulin G
JAK	Janus Kinase
LBD	ligand-binding domain
MAPK	MAP kinase
MEK	MAP kinase kinase
MNase	micrococcal nuclease
mRNA	messenger ribonucleic acid
NCoR	Nuclear receptor co-repressor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization sequence
NO	nitric oxide
NOS	nitric oxide synthase
NTD	NH ₂ -terminal domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBP	PPAR binding protein
PI3K	phosphatidylinositol-3-kinase
PMSF	phenylmethylsulphonyl fluoride
PR	progesterone receptor
PRL	prolactin
PRLR	prolactin receptor
PTEN	Phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
REA	repressor of ER activity
RNase	ribonuclease

RT	reverse transcription
SDS	sodium dodecylsulphate
SERM	selective estrogen receptor modulator
SH2	Src-homology 2
siRNA	small interfering RNA
SMRT	silencing mediator of retinoic acid and thyroid receptors
SOCS	suppressors of cytokine signalling
Sp1	Specificity protein 1
Stat	signal transducer and activator of transcription
TGN	trans-Golgi network
TEMED	tetramethylethylenediamine

Weights and measures

°C	degree Celsius
μl	microliter
μM	micromolar
bp	basepair
cm	centimetre
g	acceleration of gravity
h	hour
kbp	kilo basepair
M	molar
min	minutes
ml	millilitre
mM	millimolar
n	number
ng	nanogram
nm	nanometre
nM	nanomolar

P	probability
<i>p</i> M	picomolar
U	unit of enzyme activity
V	volt

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CHAPTER 1. INTRODUCTION

1.1 Breast Cancer

1.1.1 Statistics

Breast cancer is the most common cancer among Canadian women, excluding non-melanoma skin cancer (Canadian Cancer Society, 2012). Breast cancer occurs in both men and women, but the prevalence is low in men. In the year 2012, it was estimated that 200 men and 22,700 women would suffer from breast cancer, and 55 men and 5100 women were projected to die from it. On a daily average, 62 Canadian women are diagnosed with this disease every day and 14 of them will die. One in 9 women is expected to develop breast cancer during her lifetime and one in 29 will die of the disease (Canadian Cancer Society, 2012). The number of global cancer deaths is projected to increase by 45% from 2007 to 2030 (from 7.9 million to 11.5 million deaths), influenced in part by an increasing and aging global population (World Health Organization, 2008).

1.1.2 Risk factors

Although breast cancer is very common in North America, relatively few risk factors have been well established. Multiple lines of evidence suggest that genetic and reproductive factors are two of the better-established risk factors. Age, geographical location, obesity, lifestyle factors such as smoking, drinking alcohol and high fat diet, and hormone receptor status are some of the other established risk factors that are associated with breast cancer.

Being female and of older age are probably the two most well documented and non-modifiable risk factors for breast cancer (Reeder and Vogel, 2008). Women above

the age of 65 have 5.8 times greater risk of having breast cancer than women below the age of 65 (Singletary, 2003). For women under the age of 25, the incidence of breast cancer is very low, with only 10 out of 100,000 women being diagnosed by the disease, whereas the risk for women above the age of 45 increases by 100 fold (Dumitrescu and Cotarla, 2005). Breast cancer incidence also varies with geographical location. For example, North America and North European countries have the highest incidence of breast cancer compared to the lowest levels in Eastern Europe, South Latin America and Asia (Parkin, *et al.*, 1999) (American Cancer Society, 2008).

Reproductive factors have been well associated with breast cancer risk as early as the 17th century (Mustacchi, 1961). Nulliparous women (women who have never given birth to a viable, or live, infant) have a 20-70% increased risk of breast cancer, compared to parous women (having given birth one or more times). It was also observed that women who are above 30 years of age when giving birth to their first child have higher risk of breast cancer than younger first-time mothers (Kelsey, *et al.*, 1993).

It is well established that the exposure to endogenous sex hormones has been associated with high breast cancer risk. For instance, a younger age of menarche means prolonged exposure of the breast epithelium to estrogens and progesterones, which increases the risk factor for breast cancer by 10-20% (Bernstein, 2002; Pathak, *et al.*, 2000). Similarly, women who are menopausal after the age of 55 have an increased risk of breast cancer, compared to women who reach menopause below the age of 45, which is also attributed to longer exposure to endogenous sex hormones (Singletary, 2003). Not surprisingly, the use of exogenous hormones after menopause also increases the risk of breast cancer, and is dependent upon the duration of exposure and whether estrogen was

used with progestins (Ross, *et al.*, 2000). Likewise, hormone replacement therapy and the use of oral contraceptives increase breast cancer risk (Colditz, *et al.*, 2000; Veronesi, *et al.*, 2005). Therefore, treatments with hormone replacement therapy have decreased in the US, which is often used to explain the corresponding 9% decrease in breast cancer incidence observed over the same period (Coombs, *et al.*, 2010).

Genetic predisposition has long been established as a critical risk factor for breast cancer. Inherited mutations, such as in BRCA1 and BRCA2 genes, lead to high-risk genotypes associated with up to 80% lifetime risk of developing breast cancer (Kenemans, *et al.*, 2004; Narod, *et al.*, 2006). Although, mutations in BRCA1 and BRCA2 only account for approximately 5-10% of all breast cancers (Campeau, *et al.*, 2008), they account for 80-90% of familial breast cancers (de Jong, *et al.*, 2002).

1.1.3 Therapies for Breast Cancer

The major treatment methods used to eliminate or reduce breast tumours are primary and adjuvant therapies. Local or primary therapy involves surgery, which includes mastectomy, when the whole breast is surgically removed, or lumpectomy, when a small amount of tumour tissue is removed. Radiation therapy is also a common primary therapy against breast cancer (National Cancer Institute, 2009).

The majority of patients with early stage breast cancer also receive adjuvant therapy to increase the chances of disease-free survival. Adjuvant therapies are generally systematic, that is, they are administered through the bloodstream to reach the cancer cells and kill them. Adjuvant therapies include endocrine therapy, chemotherapy, tissue-targeted therapies, or a combination of treatments (National Cancer Institute, 2009).

Tissue-targeted therapies usually target a specific molecule that is involved in apoptosis, cell cycle control, angiogenesis, cell invasion or metastasis of the tumour cell (National Cancer Institute, 2009).

Endocrine therapy (also called hormone/hormonal therapy) reduces or eliminates the growth of hormone-sensitive tumours by inhibiting the production of these hormones by the body or by interfering with hormone receptor action. Therefore, the use of endocrine therapy depends upon the hormone receptor status of cancer cells, and typically refers to the presence or absence of the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (Her2/neu). The presence of the ER in breast cancer is associated with low-grade tumours whereas its absence has been correlated to aggressive tumours (Knight, *et al.*, 1977). ER-positive tumours are treated with anti-estrogens, tamoxifen and raloxifene (Fisher, *et al.*, 1998; Ross, *et al.*, 2000). The use of a selective estrogen receptor modulator (SERM) like tamoxifen, that blocks the action of the ER, is one of the most practiced endocrine therapies (Lewis and Jordan, 2005). PR-positive tumours are treated with anti-progesterone like mifepristone (Klijn, *et al.*, 2000). Her2-positive tumours are treated with monoclonal antibody trastuzumab (Herceptin) (Molina, *et al.*, 2001).

In addition to the inhibition of ER action, several other strategies have been developed to treat estrogen-sensitive breast cancers. These generally involve the inhibition of ovarian function in order to block estrogen production. Since, the ovaries are the main sources of estrogen, blocking ovarian function by surgically removing the ovaries (ovaries ablation) is one way of eliminating estrogen production. Secondly, ovarian function can be blocked by using drugs like gonadotropin-releasing hormone

(GnRH) agonist, which interferes with pituitary signals that stimulate the ovaries to produce estrogen (National Cancer Institute, 2012). Thirdly, aromatase inhibitors, like anastrozole and letrozole, are used to inhibit the enzyme aromatase, which converts androgens to estrogens in the ovary and other tissues (National Cancer Institute, 2012).

In contrast to hormone sensitive tumours, hormone-insensitive tumours (e.g., triple negative breast tumours) do not respond to endocrine therapy (National Cancer Institute, 2012). However, these triple negative breast tumours could express other hormone receptors like the prolactin receptor (PRLR) and androgen receptor (AR), each of which could play a role in the growth and progression of breast tumours. Traditional therapies are aimed at blocking the action of ER, PR and Her2/neu but, to date, the PRLR and AR have received little recognition in endocrine therapies (see section 1.3.4 and 1.4.3 for PRLR and AR role in breast cancer).

1.2 Estrogen

The three major types of naturally occurring estrogen are estrone, 17β -estradiol (E2), and estriol. Estradiol is the predominant estrogen during the reproductive years, both in terms of absolute serum levels as well as in terms of estrogenic activity (North American Menopause Society, 2007). As mentioned above, estrogens are primarily synthesized in the ovaries of females. They are released by the ovarian follicles, and are also secreted by the corpus luteum, under the stimulation of luteinizing hormone and follicle-stimulating hormone (Dowsett, *et al.*, 2005). Males synthesize estrogens in their testes. Like other steroid hormones, the major biosynthetic pathway for estrogen begins with cholesterol, which is converted to androgen in multiple steps. The enzyme aromatase catalyzes the last step, the aromatization of androgens into estrogens.

Therefore, androgens produced by both the adrenals and ovaries of females, or by the adrenals of males, become the primary sources of estrogen (Dowsett, *et al.*, 2005).

Estrogen synthesis also occurs in normal breast tissues and breast carcinomas (Perel, *et al.*, 1981; Suzuki, *et al.*, 2003). The local estrogen synthesis might be of particular importance in relevance in carcinogenesis.

1.2.1 Estrogen Receptors

Jensen and Jacobson for the first time proved that the biological effect of estrogen is mediated by a receptor protein called the estrogen receptor (ER) (Jensen and Jacobson, 1962). The mechanism of action of the ER is similar to other members of the nuclear receptor superfamily.

Two genetically distinct forms of the receptor, ER α and ER β , which are encoded by two different genes, mediate ER signalling. Both forms are members of the nuclear receptor superfamily, and share a common structure in that they are composed of three independent, but interacting, functional domains. These domains are the NH₂-terminal domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD). The two ER forms share a high degree of sequence homology (except in the NTD), possess similar affinities to the ligand, and recognize the same cis-acting elements called estrogen response elements (EREs) to regulate target genes (Heldring, *et al.*, 2007). The domain that follows the DBD is the hinge region, which contains the nuclear localization sequence (NLS). After the binding of the ligand to the ER, conformational changes take place in the receptor leading to the unmasking of the NLS region. ER α and ER β have also

been shown to form hetero dimers on EREs to regulate gene transcription of ER-regulated genes (Cowley, *et al.*, 1997).

1.2.2 Signal Transduction of the ER

In the absence of ligand, the ER is inactive and sequestered in the nuclei. The ligand-bound ER undergoes conformational changes within the nucleus, undergoes homo/hetero-dimerization and exhibit high affinity binding to the specific EREs, and to regulate gene transcription through interaction with coregulators and recruitment of the transcriptional machinery (Smith, *et al.*, 2004). The consensus sequence of the ERE has been determined to be 5'-GGTCAnnnTGACC-3' (Klein-Hitpass, *et al.*, 1986). Gene promoters that contain this unique sequence and that are modulated by 17 β -estradiol, include that which encode ER, PR, TGF α , pS2, c-MYC, c-FOS, cathepsin-D (Jakowlew, *et al.*, 1984; Kamalakaran, *et al.*, 2005; Morisset, *et al.*, 1986). Initiation of transcriptional activity requires interactions between transcription factor, co-activators and co-repressors. The co-activators required for ER activity include SRC1/SRC2, p68, p300, CREB binding protein (CBP), SWI/SNF, calmodulin and PPAR binding protein (PBP) (Klinge, 2000). The interactions of the ligand-bound ER with its co-activators trigger the transcription of ER-regulated genes. Similarly, interactions of the liganded ER with co-repressors inhibit the transcription of ER-regulated genes. Some of the known co-repressors of the ER include nuclear receptor co-repressor (NCoR), silencing mediator of retinoic acid and throid receptors (SMRT), and repressor of ER activity (REA) (Nilsson and Gustafsson, 2000).

The ER is a ligand-dependent transcription factor, which, upon the binding of ligand modulates the transcription of genes whose promoters contain the EREs. For many years, ER-ERE binding was thought to be the only mechanism by which ER can modulate target gene transcription. However, today it is known that the ER can modulate its target genes without binding to DNA. One example of such a non-genomic action of ER is the physical interaction between ER α and transcription factor Specificity protein 1 (Sp1), which directly activates ER-target genes (Batistuzzo de Medeiros Silvia R, 1997). Another example is the interaction between ER α and the c-rel subunit of the transcription factor NF- κ B complex, which prevents NF- κ B from binding to the interleukin-6 promoter, thereby suppressing the expression of this cytokine (Galien and Garcia, 1997).

Several ER target genes do not contain consensus EREs, but they contain ERE-half sites or GC-rich regions (Dubik and Shiu, 1992). The ligand-bound ER can also activate its target genes by binding to non-consensus ERE motifs. For example, E2 induction of cathepsin-D gene transactivation requires a GC rich region and ERE half-sites in the promoter. Similarly, the gene promoters of PR, c-MYC and creatine kinase B (CKB) each require an ERE half-site to mediate ER-activated gene transcription (Petz, *et al.*, 2004; Safe, 2001).

1.2.3 Estrogen, Estrogen Receptor and Breast Cancer

During the last five decades, we have moved from the thought that postmenopausal estrogen has the potential of reducing breast cancer risk to the current scenario where a plethora of studies suggest a direct relationship between postmenopausal estrogen levels and breast cancer risk. Epidemiologic and experimental

data implicate estrogen, in addition to other genetic factors (BRCA 1, BRCA 2, PTEN or LKB1 mutation), in breast cancer (Martin and Weber, 2000). For example, a study compared estradiol levels between healthy postmenopausal women and postmenopausal women with breast cancer. Higher levels of 17β -estradiol or estrone were present in the women with breast cancer (Key and Pike, 1988).

Despite extensive study, the molecular mechanisms by which estrogen can influence breast cancer risk and development are not completely understood. The most widely accepted concept on the role of estrogen in breast cancer development is that estrogen, acting through $ER\alpha$, has the ability to stimulate cell proliferation, and initiate mutations arising from errors in DNA replication (genetic errors). Estrogens stimulate the growth of cells that carry these mutations, which then increases the risk of breast cancer (Pike, *et al.*, 1993; Preston-Martin, *et al.*, 1990). A study by Clarke and his group showed that $ER\alpha$ levels increase markedly in breast carcinogenesis, but growth of these cells are inhibited by antiestrogens (Clarke, *et al.*, 1993). The same group demonstrated that $ER\alpha$ levels in proliferating breast cancer cells are higher than in epithelial cells of the normal mammary gland (Clarke, *et al.*, 1997), further supporting the roles of estrogen and $ER\alpha$ in breast cancer development. The ER mediated crosstalk with other signalling pathways can also contribute to breast cancer cell survival. For example, insulin like growth factor-2 (IGF-2) activated insulin like growth factor 1-receptor (IGF-1R) promote the phosphorylation and translocation of $ER\alpha$ and $ER\beta$ to the nucleus, plasma membrane and mitochondria leading to the activation of cell survival pathways. This pathway could be of particular importance in breast cancers that grow independent of estrogen (Richardson, *et al.*, 2011).

Estradiol and its metabolites can also exert ER-independent DNA damage (Fernandez, *et al.*, 2006; Kong, *et al.*, 2000), contributing to the development of breast cancer. Wei Yue and his colleagues demonstrated that exogenous estradiol accelerated tumour formation in a dose-dependent fashion in ER α knockout mice. In contrast, the reduction of endogenous estrogen by aromatase inhibitor resulted in delayed tumourigenesis even in ER α knockout mice, suggesting ER-independent action of estrogen in the development of breast cancer (Yue, *et al.*, 2010). The inhibition of estrogen action by tamoxifen or raloxifene reduces the breast cancer risk by 50-70% in high-risk women (Cummings, *et al.*, 1999; Cuzick, 2001). Two other independent studies have demonstrated that inhibition of estrogen action by antiestrogens or inhibition of estrogen synthesis by aromatase inhibitors prevents the development of breast cancer during adjuvant therapy (Howell, *et al.*, 2005; Thurlimann, *et al.*, 2005). To date, blockade of ER action is the most common therapy for ER positive breast cancer. However, ER-independent effects of estrogen and estrogen-independent effects of ER can also influence breast cancer development in concert with estrogen activated ER-dependent effects. All of these studies, when put together, provide strong evidence that estrogen plays a major etiologic role in the development of breast cancer.

1.3 Prolactin

Prolactin (PRL) is a polypeptide hormone synthesized and secreted by the anterior pituitary gland (Stricker and Greuter, 1928). Originally, this hormone was characterized by its ability to promote lactation and mammary gland development, hence the name prolactin (Freeman, *et al.*, 2000). However, we now know that PRL has over 300 diverse biological activities (Bole-Feysot, *et al.*, 1998) and cannot be defined by its name.

Furthermore, we are now aware that the synthesis and secretion of PRL is not confined to the anterior pituitary gland. Extrapituitary sites such as the mammary gland, prostate gland, brain, thymus, spleen, and immune cells express the PRLR, and thus, PRL may act as an autocrine/paracrine growth factor in these tissues (Ben-Jonathan, *et al.*, 1996; Bern and Nicoll, 1968; Freeman, *et al.*, 2000; Harris, *et al.*, 2004). Pituitary PRL acts via a classical endocrine pathway where it is transported by the circulation to peripheral tissues that express the PRLR on the cell plasma membrane. On the other hand, locally produced PRL can act on peripheral tissues (paracrine) or on the tissues producing it (autocrine).

The gene encoding human PRL (hPRL) is located on chromosome 6 (Owerbach, *et al.*, 1981). The hPRL gene is more than 15 kbp in length and is composed of 5 coding exons separated by 4 introns (Truong, *et al.*, 1984). An extra non-coding exon is present in the hPRL gene in extra-pituitary sites (Gellersen, *et al.*, 1989). The proteolytic processing of pro-hormone PRL produces the mature hPRL, comprised of 199 amino acids and with a total molecular mass of 23 kDa (Cooke, *et al.*, 1981).

1.3.1 Pituitary and Extrapituitary Prolactin Gene Expression

The PRL gene promoter has been characterized in rat and human. In the rat PRL gene promoter, a distal promoter and a distal enhancer region have been identified. These two regions are highly conserved in rat and human (Peers, *et al.*, 1990). In the human PRL gene promoter, a superdistal enhancer region has also been identified but its function has yet to be determined (Van De Weerd, *et al.*, 2000). In both rat and human, pituitary PRL expression is dependent upon transcription factor Pit-1. PRL gene

expression is also regulated by cytokines, steroids, as well as by other hormones such as insulin (Ben-Jonathan, *et al.*, 1996; Gourdjji and Laverriere, 1994).

The cell-specific gene expression profile of the PRL gene in extra-pituitary sites has been reported in rats and humans. Unlike the pituitary hPRL gene promoter, the extra-pituitary promoter exhibits Pit-1-independent activity and responsiveness to different regulators of gene expression (Gellersen, *et al.*, 1994). For example, progesterone and insulin exert cell type-specific effects on extra-pituitary prolactin expression (Ben-Jonathan, *et al.*, 2008; Featherstone, *et al.*, 2012; Hugo, *et al.*, 2008).

1.3.2 PRL Receptor

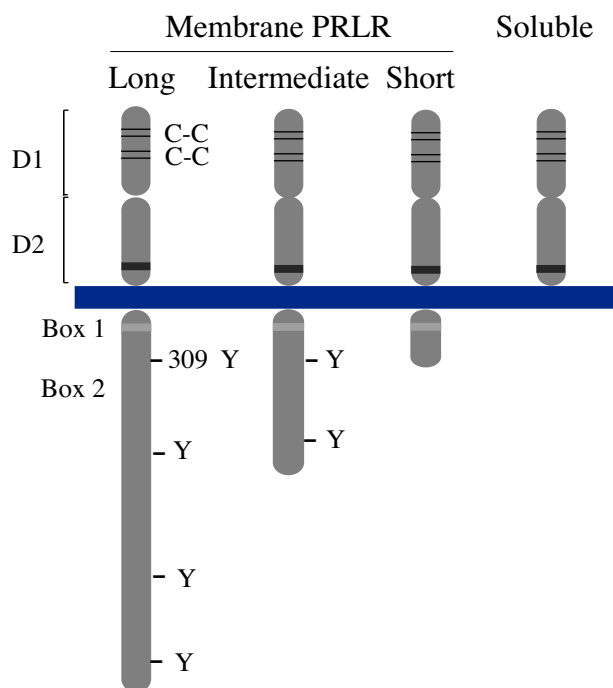
The PRL receptor (PRLR) was identified as a specific, high affinity, membrane-anchored protein (Posner, *et al.*, 1975) that belongs to the class I cytokine receptor superfamily (Kelly, *et al.*, 1991). Other members of this family include receptors for growth hormone, granulocyte macrophage-colony stimulating factor, several interleukins, erythropoietin, and the obesity factor leptin (Bazan, 1990; Bole-Feysot, *et al.*, 1998; Boutin, *et al.*, 1989; Cosman, 1993). PRLR and GH receptor are both single transmembrane chains and share structural and functional features, despite low degree sequence identity (~ 30%) (Goffin and Kelly, 1996; Kelly, *et al.*, 1991). Some cytokine receptors also share common structural motifs with the PRLR. Therefore, in addition to PRL, primate growth hormone and placental lactogens can bind to the hPRLR to activate signalling cascades specific to the receptor (Goffin, *et al.*, 1996).

The gene encoding the hPRLR is located in chromosome 5 and contains at least 10 exons spanning over 100 kbp in length (Arden, *et al.*, 1990; Boutin, *et al.*, 1989). The

PRLR is distributed widely in many tissues and cell types, and numerous isoforms have been identified in humans and rats tissues (Bole-Feysot, *et al.*, 1998). The multiple isoforms of the PRLR is the result of alternative splicing of the primary transcript. PRLR isoforms vary in length and cytoplasmic domain compositions, but the extracellular domains are identical. The various isoforms are referred to as short (291 aa), intermediate (393 aa) and long PRLR (591 aa), depending upon the length of the cytoplasmic tail (Bole-Feysot, *et al.*, 1998) (see Figure 1).

In mice, one long and three short PRLR isoforms has been cloned (Davis and Linzer, 1989). In addition to the membrane-anchored PRLR, soluble prolactin-binding isoforms were described in mammary epithelial cells (Berthon, *et al.*, 1987). The soluble PRLR isoform is a PRLR gene product, but whether they are the results of alternative splicing of the primary transcript or proteolytic cleavage of membrane-anchored PRLR (mature receptor) or both is uncertain (Amit, *et al.*, 1997; Fuh and Wells, 1995; Postel-Vinay, *et al.*, 1991).

In humans, six PRLR isoforms have been cloned. The long hPRLR isoform migrated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at approximately 85 kDa (Bazan, 1990). The intermediate form lacks a 198 aa region that forms the cytoplasmic tail, due to alternative splicing, and migrates in SDS-PAGE at about 50 kDa (Ali, *et al.*, 1992; Kline, *et al.*, 1999). The intermediate form was first cloned in pre-T rat lymphoma Nb2 cell line, which is dependent on PRL for mitogenesis (Ali, *et al.*, 1992). The short isoform of the hPRLR also has a truncated cytoplasmic tail and is approximately 36 kDa (Kline and Clevenger, 2001).



	Long	Intermediate	Short	Soluble
Length (aa):	591	393	291	206
Size (kDa):	85	50	36	32
Jak/Stat pathway activation :	Yes	Yes	Yes	No

Figure 1. Schematic Representation of PRLR Isoforms (adapted from Christine Bole-Feysot et al., 1998 with modifications). The extracellular domain of the receptor contains two type III fibronectin-like domains (D1 and D2) that are responsible for binding of ligand. The Box 1 and Box 2 motifs characterize the membrane proximal region of the cytoplasmic domain that is highly conserved in the cytokine receptor superfamily. Proline-rich Box 1 is required for Jak2 binding.

1.3.3 Activation of Prolactin Receptor Signalling

The PRLR contains three domains, which partition the receptor into an extracellular domain (ECD), a transmembrane domain and an intracellular domain. The ECD of PRLR is composed of ~200 amino acid region that has sequence similarity with other cytokine receptors and is referred to as cytokine receptor homology domain (CRH) (Finidori and Kelly, 1995). The CRH domain can be divided into two ~100 amino acid subdomains, namely NH₂-terminal D1 and membrane-proximal D2. These conserved subdomains D1 and D2 demonstrate analogies with the fibronectin type III molecule, which is responsible for mediating receptor-ligand interactions (Wells and de Vos, 1996). The transmembrane domain of the PRLR is 24 amino acid long (Bole-Feysot, *et al.*, 1998). The intracellular domains are different in length and composition across different isoforms of PRLR, but they have two relatively conserved regions referred to as Box 1 and Box 2. Box 1, a proline-rich motif, is required for the consensus folding of the molecule recognized by the transducing molecules. Box 2 is less conserved and is missing in the short PRLR isoform (Goffin, *et al.*, 1998).

Ligand-mediated activation of the PRLR takes place when the ligand (PRL, placental lactogens or growth hormone) is bound to the receptor. The formation of an active receptor-ligand complex initiates receptor-associated intracellular signalling pathways. The binding of ligand triggers receptor dimerization, (Gertler, *et al.*, 1996), which in turn induces phosphorylation of Janus Kinase 2 (Jak2). Jak2 is found to be constitutively associated with PRLR (Campbell, *et al.*, 1994). Jak2s trans-phosphorylate each other, and are also involved in phosphorylation of tyrosine residues of the PRLR, namely Tyr309 and Tyr382. The phosphorylated tyrosine residues, serve as docking sites

for the Src-homology 2 (SH2) domain of the Stat proteins (Signal transducer and activator of transcription), particularly Stat1, Stat3 and Stat5 (Bole-Feysot, *et al.*, 1998). The monomeric Stat proteins, recruited to the active receptor, are then phosphorylated by Jak2. The active Stats dissociate from the receptor, homo or heterodimerize through the interaction between a phosphorylated tyrosine of one Stat and the SH2 domain on another Stat. The dimerized Stat complex then translocates to the nucleus where it binds to a specific DNA motif called γ -interferon activated sequences (GAS) in the promoter region of target genes (Clevenger, *et al.*, 2003), such as cyclin D1 (Brockman, *et al.*, 2002), interferon 1, and milk protein genes (such as β -casein and lactoglobulin) (Yu-Lee, *et al.*, 1990) (see Figure 2). Numerous gene promoters that contain the GAS motif, comprising a palindromic sequence TTCxxxGAA, are regulated by PRL (Ferrag, *et al.*, 1996).

All Stat proteins contain a DNA-binding domain, a SH3-like domain, a SH2 domain, an ubiquitous tyrosine and a C-terminal trans-activating domain (Finidori and Kelly, 1995). Among the three Stat proteins that are known to be activated by PRL, Stat5 is considered the most important transducer of the PRLR long and intermediate isoforms (Liu, *et al.*, 1995). Stat5 has two isoforms, Stat5a and Stat5b, which are encoded by different genes. These isoforms are 96% conserved at the protein level (Koptyra, *et al.*, 2011), with the major differences lying in the C-terminal domain. Both Stat5 isoforms possess the functionally essential tyrosine residue (Tyr-694) that is phosphorylated by Jak2 (Gouilleux, *et al.*, 1995). The finding that a PRLR mutant is unable to activate Jak2 and Stat5 is in consensus with the finding that Stat5 acts as the major transducer of signals from the PRLR (DaSilva, *et al.*, 1996; Pezet, *et al.*, 1997). The activation of Stat5 by Jak2 is inhibited by a SH2-containing family of proteins, referred as cytokine-

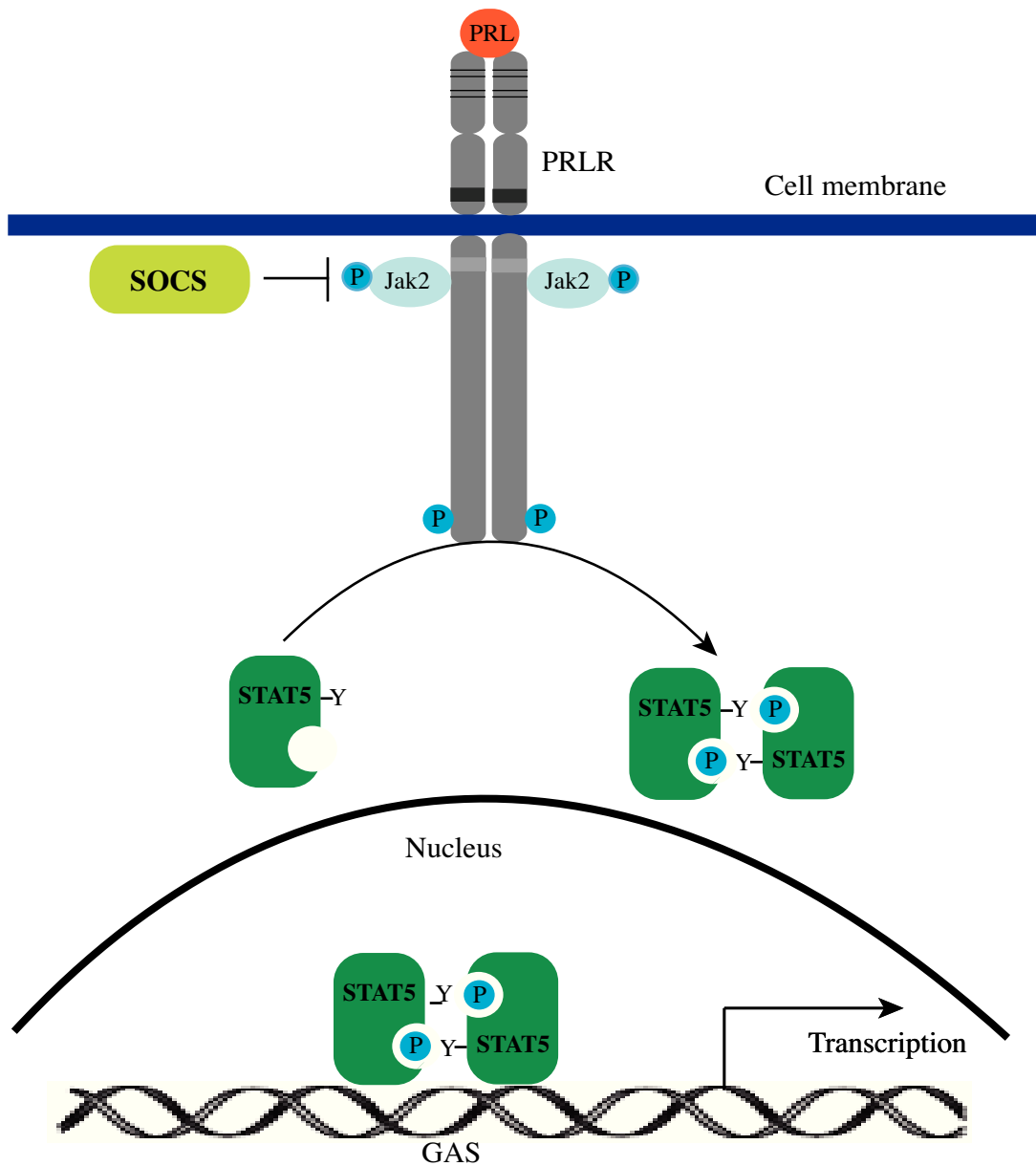


Figure 2. Schematic Representation of PRL-Jak2-Stat5 pathway (adapted from Ke Shuai & Bin Liu et al., 2003 with modifications). PRL, bound to the dimerized PRLR, activates Jak2 kinase which is associated with the PRLR. Jak2 auto-phosphorylates the receptor to create a docking site for transcription factor Stat5. Before its activation, Stat5 is found in the cytoplasm as a monomer. Stat5 is phosphorylated by receptor-associated Jak2 while docked at the receptor. Upon activation, Stat5 forms a homo/heterodimer (between Stat5a/Stat5a or Stat5a/Stat5b). Stat5 dimers then translocate to the nucleus where they bind to GAS (γ -activated sequence) elements in target genes. SOCS proteins, which are induced by cytokines, act as a negative feedback mechanism to shut off the Jak2 kinase activity.

inducible SH2-containing protein (CIS) and suppressors of cytokine signalling (SOCS). CIS and SOCS inhibit cytokine signalling by competing with Stat5 binding to the receptor (CIS) or by interacting with Jak kinases (SOCS) (Pezet, *et al.*, 1999).

Although the Jak-Stat pathway is the most important pathway involved in cytokine receptor signalling, other signal transducing pathways such as MAP Kinase (MAPK) is also activated following PRL stimulation. PRL activation of Raf1 serine/threonine kinase, MAPK, and MAP kinase kinase (MEK) has been reported in several cellular systems (Das and Vonderhaar, 1996; Piccoletti, *et al.*, 1994). The activation of Stat proteins can be modulated by other mediators, including MAPK, which can regulate the activity of Stat5 through serine phosphorylation (Decker and Kovarik, 2000; Yamashita, *et al.*, 1998). Although the Jak-Stat and MAPK pathways are considered independent, there are instances when these two pathways are interconnected to modulate transcription of PRL-responsive genes (Ihle, 1996).

1.3.4 PRL and Breast Cancer

The role of PRL in the initiation and progression of rodent mammary carcinoma has been clearly established (Mershon, *et al.*, 1995). Wennbo and his colleagues demonstrated a direct correlation between increased PRL secretion and tumourigenesis in mice. Transgenic female mice that overexpressed the rat PRL gene spontaneously developed mammary carcinomas at 11 to 15 months of age, but the bovine growth hormone transgenic mice and control mice did not (Wennbo, *et al.*, 1997). In contrast, the role of PRL in human breast cancer has been historically controversial, and it has been difficult assigning a role for PRL in the etiology and progression of human breast cancer.

Bromocriptine treatment, to inhibit pituitary PRL synthesis and secretion, had no effect on human breast cancer. However, the lack of an effect can be attributed to the fact that bromocriptine does not inhibit extrapituitary PRL synthesis and secretion (Muthuswamy, 2012).

More than 90% of the normal human breast tissues and breast cancer biopsies are positive for PRL and its receptors. Almost 80% of the breast cancer cells in culture respond to the mitogenic signal of PRL under reduced serum conditions (Das and Vonderhaar, 1996). Several epidemiological studies have suggested a role for PRL in the progression of human breast cancer. In a large study conducted by Hankinson and her colleagues, blood samples were collected and archived from 32,826 nurses. In a 5-year follow up they identified 306 breast cancer cases and 448 controls, and had the women's PRL levels measured. The investigators found a statistically significant positive association between the plasma levels of PRL and the breast cancer risk. Women with higher plasma PRL levels had higher risk of breast cancer, relative to women with lower plasma levels (Hankinson, *et al.*, 1999). In another study, 44% of patients with metastatic breast disease were found to be hyperprolactinemic (elevated serum prolactin) during the course of the disease (Holtkamp, *et al.*, 1984). Increased levels of PRL were observed in postmenopausal women with increased breast tissue density (Wang, *et al.*, 1995), suggesting an influence of PRL on breast epithelial and/or stromal proliferation.

In vitro studies of breast cancer tissues show a clear response to different levels of PRL. Biswas & Vonderhaar showed that PRL-stimulated growth of MCF-7 breast cancer cells is more evident in 1% charcoal-stripped serum than in 10% charcoal-stripped serum. Growth effects were seen at concentrations as low as 25 ng/ml hPRL and the maximal

effect was observed at 100-250 ng/ml (Biswas and Vonderhaar, 1987). There are evidences that physiological levels of hPRL stimulate the growth of mammary epithelial cells (Imagawa, *et al.*, 1985) and primary breast tumour biopsies in culture (Malarkey, *et al.*, 1983). The T47D and MCF-7 breast cancer cell lines respond to the PRL growth signal when cultured as solid tumours in nude mice. PRL-neutralizing antibodies and PRLR-specific antagonist (Δ G129hR-hPRL) were shown to inhibit PRL-induced proliferation of several breast cancer cell lines, including MCF-7 and T47D (Fuh and Wells, 1995). In cell lines derived from MCF-7 cells that do not express PRL endogenously, exogenous PRL has been shown to mediate cell cycle progression by induction of cyclin D1, a critical cell cycle regulator (Schroeder, *et al.*, 2002).

Schroeder and his colleagues demonstrated that the administration of PRLR antagonist Δ G129hR-hPRL induces apoptosis in T47D cells, suggesting a role for PRL in cell proliferation (Schroeder, *et al.*, 2002). PRL, acting via phosphatidylinositol-3-kinase (PI3K) dependent mechanisms stimulates cellular motility, an important factor in tumour cell progression (Maus, *et al.*, 1999). Another study showed that breast cancer cells responded to PRL-neutralising antibody with the induction of apoptosis, suggesting that endogenous PRL was crucial for cell survival. The same group also showed that PRL protects the cell from undergoing ceramide-induced apoptosis (Perks, *et al.*, 2004). Despite the accumulated evidence for the role of PRL in breast carcinogenesis, the PRLR is not a target for conventional endocrine therapy.

1.4 Androgen

Androgens are male sex steroids that have many physiological functions, including development of the male accessory sex organs and male secondary sex

characteristics. Androgens are produced by the testes in males, the ovaries in females and by the adrenal gland in both sexes (Park, *et al.*, 2010). Testosterone is the principal circulating androgen secreted by testicular leydig cells following stimulation by luteinizing hormone. Another androgen is dihydrotestosterone (DHT), which is more potent than testosterone in its androgenic activity. DHT mainly functions in the virilization of the external genitalia in males, leading to the proper differentiation of the prostate, urethra, penis and scrotum. In addition, DHT plays a role in the development of secondary sexual characteristics such as muscle building and bone mass. The adrenal gland also secretes dihydroepiandrosterone and androstenedione, both of which function as weak androgens with only about 5-10 % potency, as compared to testosterone or DHT, and are precursors of androgens (Chawnshang, 2002). Androgens, mainly testosterone and DHT, exert most of their effects by interacting with a specific receptor, the androgen receptor (AR).

1.4.1 Androgen Receptor

The AR is a member of the ligand-activated nuclear receptor superfamily. AR, in common with other members of this superfamily, functions as a ligand-induced transcription factor. The AR has two natural ligands, testosterone and DHT, both of which when bound to the AR, activate target gene expression at the transcriptional level (Gelman, 2002).

AR is a modular protein, which is divided into four structurally and functionally distinct domains. It consists of an NH₂-terminal transactivation domain (NTD), a DNA-binding domain (DBD), ligand-binding domain (LBD), and a small hinge region, which

together mediate the genomic actions of testosterone in androgen target tissues. The structures of LBD and DBD are highly conserved across species, but NTD shows the greatest degree of variability, both in terms of sequence and length (Gelman, 2002). The AR gene was localised in the X chromosome by genetic analysis of Androgen Insensitivity Syndrome in humans and mice (Brown, *et al.*, 1989). The single copy AR gene is composed of 8 exons and spans over 90 kbp of genomic DNA (Kuiper, *et al.*, 1989). Exon 1 codes for the NTD, exons 2 and 3 code for the central DBD, and exons 4 to 8 code for the C-terminal LBD. The AR locus consists of a CpG island that spans the proximal promoter region and exon 1. The promoter lacks a typical TATA or CAAT sequence but contains GC rich elements, which is common with TATA-less promoters (Gelman, 2002).

The NTD of the AR represents about half the receptor coding sequence and is responsible for the majority of the receptor's transcriptional activity. The NTD of the AR also directly interacts with the general transcriptional machinery (Lee, *et al.*, 2000) and is the predominant site for the binding of co-activators (Alen, *et al.*, 1999). The cysteine-rich DBD contains two zinc finger motifs and a short C-terminal extension that forms part of the hinge region. The first zinc finger recognizes and interacts with the specific androgen response elements (ARE) and facilitates the binding of the AR to the major groove of DNA. The second zinc finger interacts with the first zinc finger and stabilizes the AR-DNA complex by hydrophobic interactions (Schoenmakers, *et al.*, 1999). The second zinc finger can also mediate the dimerization between two AR monomers (Dahlman-Wright, *et al.*, 1993). A hinge domain, which links DBD and LBD, consists of

a bipartite NLS and sites for phosphorylation, acetylation and degradation (Li and Al-Azzawi, 2009).

1.4.2 Molecular Mechanisms of Androgen Action

Like many other steroid hormone receptors, the AR resides in the cytoplasm, and is bound to heat-shock protein (HSP90), which prevents its degradation. The binding of androgen to the AR induces a conformational change in the receptor that causes the heat shock protein to dissociate, which allows the translocation of the liganded-AR to the nucleus, where it could undergo phosphorylation, followed by interaction with DNA (see Figure 3). The nuclear targeting of the AR complex is directed by the nuclear localization sequence in the hinge region, the mutation of which prevents the translocation of the AR complex to the nucleus (Simental, *et al.*, 1991). After binding of the ligand, the AR is phosphorylated at many sites, including S650 in the hinge region, which is required for full transcriptional activity of the AR (Zhou, *et al.*, 1995). The dimerized AR then binds to the specific AREs of the target genes and recruits the essential cofactors to initiate the regulation of androgen-responsive genes (Claessens, *et al.*, 2001; Glass and Rosenfeld, 2000). The consensus ARE is a 15-bp palindromic sequence that consists of two hexameric half sites (5'-AGAACA-3') arranged as inverted repeats with a 3-bp spacer in between (5'-GGTACAnnnTGTTCT-3'). However, in target genes, the binding site can deviate considerably from the consensus sequence. AR action is regulated by its co-regulators, which can influence ligand selectivity and DNA-binding capacity of the AR (Glass and Rosenfeld, 2000). AR action can also be influenced by other transcription factors. The binding sites for steroid receptors are often found in clusters with the binding

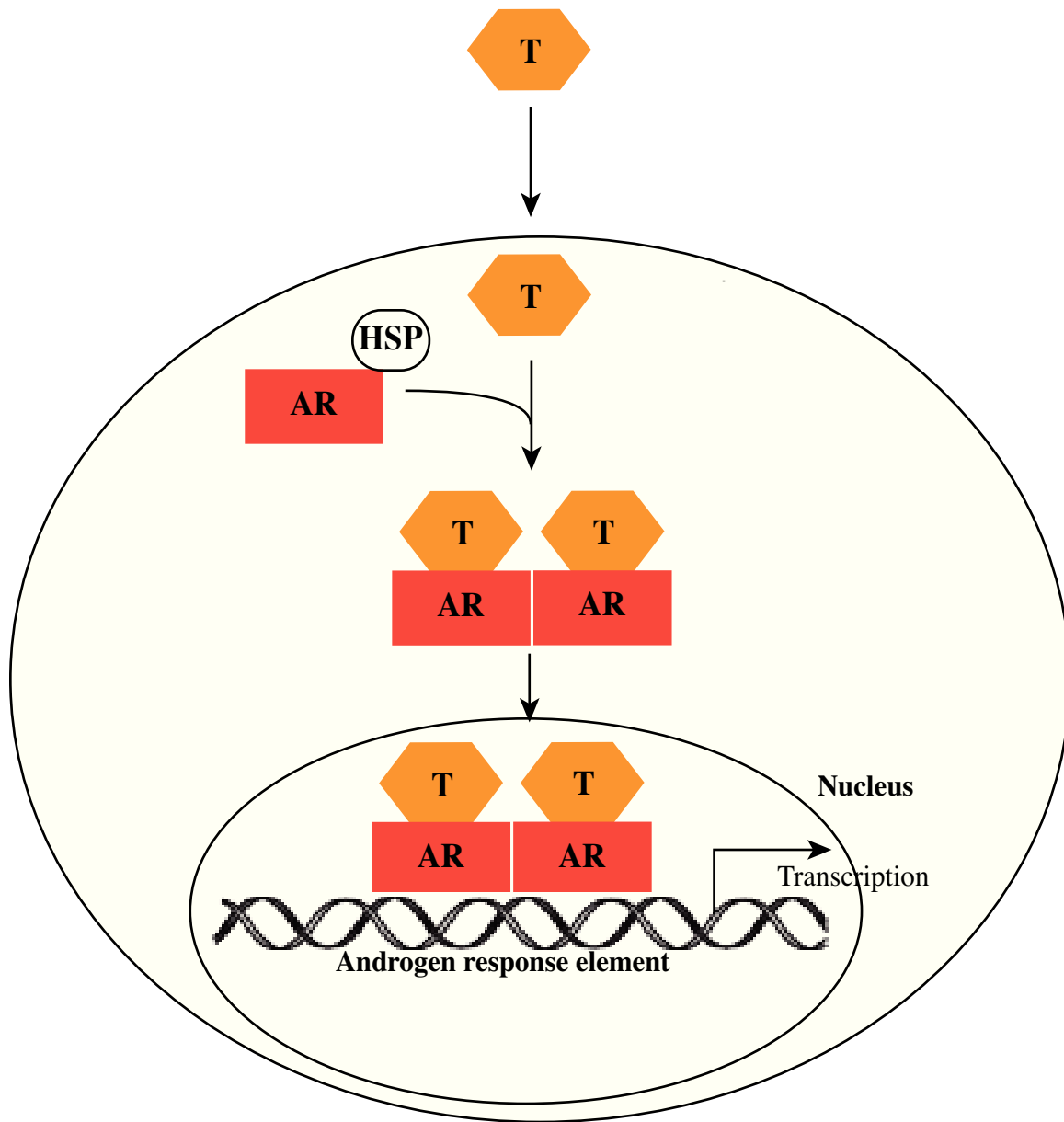


Figure 3. Schematic Representation of AR Pathway. The androgen receptor (AR) found in the cytoplasm is bound to heat shock protein (HSP) which prevents AR degradation. Binding of androgen to AR causes a conformational change in the receptor, releasing HSP. Upon activation, the liganded receptors forms a dimer. The AR dimer translocates to the nucleus, binds to androgen response elements, and activates target genes.

sites of other transcription factors. Many of these transcription factors can synergistically interact with steroid receptors, thereby influencing AR regulation of target genes (Schule, *et al.*, 1988).

1.4.3 Androgen, AR and Breast Cancer Risk

Many hormones are known to play critical roles in mammary carcinogenesis, which strengthens the rationale for their study to develop new anti-cancer therapies for breast cancer. Since, breast cancer is more prevalent in females than male, the study is focussed towards their predominant hormone, estrogen. However, male steroid hormones also have physiologic importance in breast development, even though their role in breast cancer progression and development is less understood.

The expression of the AR is abundant in normal mammary epithelium and in the majority of breast cancer specimens and cell lines. A determination of steroid receptor status in various grades of mammary carcinoma *in situ* and invasive carcinoma showed that when tumour grade progresses from 1 to 3, AR expression decreases from 95% to 76% in ductal carcinoma *in situ*, and 88% to 47% in invasive carcinoma. In contrast, ER expression decreased dramatically from 100% to 8% in ductal carcinoma, and to 9.5% in invasive carcinoma. Therefore, despite a decrease in the % of AR during disease progression, the AR is still abundantly present in these tissues, making the AR a potentially valuable target for new therapies against breast cancer (Moinfar, *et al.*, 2003).

Several epidemiological studies have successfully found a correlation between circulating androgens and breast cancer pathogenesis. These studies have demonstrated the increased risk of breast cancer development in postmenopausal women with high

17 β -estradiol and high testosterone levels (Cauley, *et al.*, 1999; Hankinson, *et al.*, 1998). Furthermore, the administration of androgens for the treatment of cystic breast disease has been shown to increase breast cancer risk (Veronesi and Pizzocaro, 1968). Similarly, postmenopausal women with high androgen levels are at an increased risk of breast cancer (Agoff, *et al.*, 2003). Preclinical studies conducted by Wong and his colleagues demonstrated that androgens, in addition to 17 β -estradiol, can induce breast tumourigenesis in young-adult female Noble rats, and the exposure to both hormones increases the incidence of breast carcinogenesis (Wong and Xie, 2001).

The proliferation of human breast cancer cell lines can be stimulated or inhibited by androgens *in vitro*, as it can be influenced by cell-specific differences, level of expression of cofactor and co-repressors, or structural alterations in the AR. Physiological and pharmacological concentrations of DHT stimulated the proliferation of the estrogen-responsive human breast cancer cell lines, MCF-7 and EFM-19. (Hackenberg and Schulz, 1996). However, the stimulatory effect of androgens was not limited to estrogen-responsive breast cancer cell lines. A synthetic androgen, mibolerone, was reported to induce proliferation of MDA-MB-453 cells, which is an ER- and PR-negative breast cancer cell line (Birrell, *et al.*, 1998). In contrast, pharmacological concentrations of androgen inhibited growth of the T47D breast cancer cell line (Sutherland, *et al.*, 1988). A large scale study reinforced the correlation between the expression of AR and the overall survival of breast cancer patients by demonstrating that patients with AR-negative tumours had a significantly lower response to hormone therapy and a shorter overall survival, compared to AR-positive tumours ($P < 0.001$) (Bryan, *et al.*, 1984). Similar to the PRLR, there is accumulating evidence that the AR

plays a role in breast cancer etiology but has received little attention in endocrine-related therapies for this disease.

1.5 Carboxypeptidases

Carboxypeptidases (CPs) hydrolyze one amino acid at a time from the C terminal regions of proteins and polypeptides through hydrolysis (Reznik and Fricker, 2001). The removal of one or a few amino acids from the C-terminus might not seem to have huge importance, but often it leads to significant alteration in the biological activity of the molecule (Skidgel, 1988). Based on the use of an active site serine, or zinc, the CPs can be grouped into 2 divisions: serine CPs and metallo-CPs. Serine CPs contain a catalytic group of amino acids (Ser, Asp, His) in the active site, which is characteristic of many serine proteases. CPs that use zinc in their cleavage mechanism are referred to as metallo-CPs (Skidgel and Erdos, 1998).

The metallo-CPs catalyze peptide hydrolysis by utilizing glutamic acid as a primary catalytic residue and a tightly bound zinc atom as the essential co-factor (Reznik and Fricker, 2001; Skidgel and Erdos, 1998). Many members of metallo-CPs (carboxypeptidases D, E, N and M) are enzymes and are thought to be involved in the processing of peptide precursors. Other members of the metallo-CP gene family, such as CPX-1, CPX-2 and ACLP, do not encode active enzymes (Reznik and Fricker, 2001). Based on their substrate specificity, metallo-CPs can be divided into CPA-type or CPB-type enzymes. The CPA-type enzymes preferentially hydrolyze C-terminal hydrophobic residues, whereas CPB-type enzymes only hydrolyze peptides that contain C-terminal basic residues, arginine (Arg) or lysine (Lys) (Skidgel and Erdos, 1998). The family of

serine-CPs includes lysosomal pro-X carboxypeptidase and deamidases, and metallo-CPs that belong to B-type include CPD, CPM and CPE.

1.5.1 Carboxypeptidase D

The human carboxypeptidase D (CPD) gene is ~ 88.3 kbp in length, comprising 21 exons and 20 introns (Timblin, *et al.*, 2002), and is located in chromosome 17 (Ishikawa, *et al.*, 1998; Riley, *et al.*, 1998). The CPD protein contains three tandem homologous carboxypeptidase (CP) domains, which are linked by short bridge regions, followed by a transmembrane domain, and a short 60-residue sequence that make up the cytosolic tail (see Figure 4) (Kuroki, *et al.*, 1995; Tan, *et al.*, 1997; Xin, *et al.*, 1997). The three CP domains (I, II and III) are believed to be the consequence of tandem duplications of an ancestral gene and all three domains are highly conserved across species (Kuroki, *et al.*, 1995). Domain I and II are active CPs with slightly different properties. Domain III is inactive as a result of mutation in many critical residues (Reznik and Fricker, 2001), but has retained some of the residues that are potentially involved in substrate binding (Aloy, *et al.*, 2001).

The 180-kDa membrane-bound CPD is a single-chain glycoprotein that cleaves C-terminal arginine from proteins and peptides (McGwire, *et al.*, 1997; Skidgel and Erdos, 1998; Song and Fricker, 1996). CPD has a broad distribution in mammalian tissues and organs, including the hippocampus, pituitary, ovaries, testes, spinal cord, pancreas, lung, kidney, cardiac atrium and gut (Song and Fricker, 1996; Xin, *et al.*, 1997). CPD is found in the *trans*-Golgi network (TGN) but significant amounts are also trafficked to the plasma membrane (Hadkar and Skidgel, 2001). CPD is also found in the nuclei of MCF-7 cells (O'Malley, *et al.*, 2005). In addition, a novel nuclear-targeted CPD

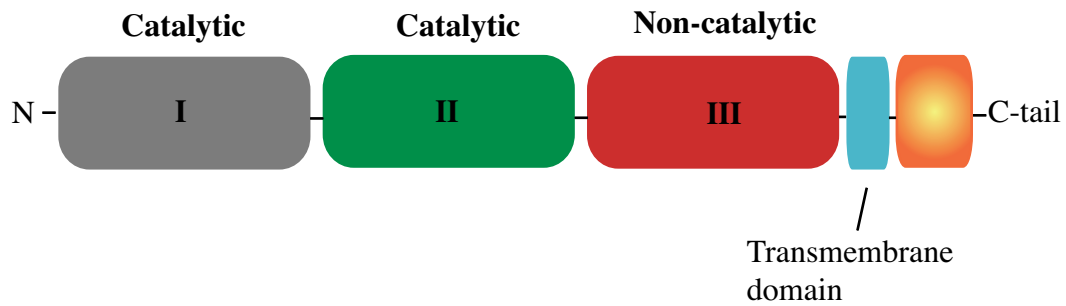


Figure 4. Domain structure of CPD. CPD is composed of three homologous extracellular carboxypeptidase domains (I, II and III), a transmembrane anchor, and a highly conserved cytoplasmic tail.

isoform (CPD-N) was identified in the rat PRL-dependent Nb2 and PRL-independent Nb2-Sp T-lymphoma cell lines (Too, *et al.*, 2001). CPD-N has a truncated N-terminus domain and thus, a lower molecular mass of 160 kDa (O'Malley, *et al.*, 2005). CPD-N is exclusively present in the nuclei of rat lymphoma and human hematopoietic tumour cells (Too, *et al.*, 2001).

The high concentration of CPD in the Golgi suggests its involvement in protein- and peptide- processing in the constitutive secretory pathway (Skidgel and Erdos, 1998). The plasma membrane localization of CPD suggests that it also functions as a cell-surface enzyme. Characterization of CPD as a functional cell-surface enzyme was pursued in studies using a mouse macrophage cell line. In macrophages stimulated with interferon- γ and lipopolysaccharide, the addition of a CPD-specific extracellular substrate stimulated nitric oxide (NO) production by six fold as a result of the CPD-mediated release of Arg from the CPD substrate (Hadkar and Skidgel, 2001). Furthermore, CPD mRNA and protein levels were increased by interferon- γ and lipopolysaccharide in macrophage cells, cultured in Arg-free medium (Hadkar and Skidgel, 2001). Arg, released by CPD, is the substrate of nitric oxide synthase (NOS). CPD induction of NO production, by cleaving C-terminal Arg from synthetic CPD substrates has also been reported in rat micro-vascular endothelial cells (Hadkar, *et al.*, 2004).

PRL stimulation of CPD mRNA expression in cancer cells was first observed in human HepG2 hepatoma and MCF-7 breast cancer cell lines (Too, *et al.*, 2001). PRL and the cytokine interleukin-2 also stimulated the expression of the nuclear CPD-N in rat lymphoma cells (Too, *et al.*, 2001). PRL has also been shown to stimulate NOS expression, which increased NO production, promoting cell survival and/or inhibition of

apoptosis in PRL-dependent rat lymphoma cells (Dodd, *et al.*, 2000) and in MCF-7 cells (Abdelmagid and Too, 2008). However, PRL stimulation of NO production was abrogated by small interfering RNA targeting CPD (siCPD), indicating that CPD, not NOS, was the major contributor of intracellular NO (Abdelmagid and Too, 2008). Similarly, PRL and testosterone upregulated CPD levels and increased NO production in several prostate cancer cell lines. The stimulation of CPD expression by PRL and testosterone suggests the presence of active Stat5a/b and AR binding sites in the CPD gene promoter (Thomas, *et al.*, 2012).

1.6 Nitric Oxide

Nitric oxide (NO) is a diatomic, highly reactive free radical molecule, and is a gas at room temperature. In mammalian cells, the three NOS isoforms, neuronal, endothelial, and inducible, catalyse the production of NO from L-Arg, and requires NADPH and oxygen as cofactors (Marletta, 1988). NOS isoforms are differentially regulated at transcriptional, translational and post-translational levels. The activities of nNOS and eNOS are highly dependent upon intracellular calcium concentration whereas calcium-independent iNOS forms an active complex with calmodulin (Alderton, *et al.*, 2001).

Over the past two decades, it has been clear that NO regulates a variety of important physiological and pathological processes. Originally, NO was identified as an endothelium-derived relaxing factor (EDRF) for its role in the cardiovascular system (Furchgott and Zawadzki, 1980; Ignarro, *et al.*, 1987; Palmer, *et al.*, 1987). NO can readily pass through membranes (Pance, 2006), and upon its release can exert its physiological effects by binding to a heme group within guanylate cyclase-coupled receptors, triggering receptor activity. Activated guanylate cyclase leads to the generation

of cyclic GMP (cGMP) from GTP. Many physiological processes that are known to be initiated or promoted by NO, including smooth-muscle relaxation and inhibition of platelet aggregation are mediated by the NO-cGMP signalling pathway (Friebe and Koesling, 2003). The cGMP-dependent protein kinases, cyclic-nucleotide-gated ion channels and cGMP-regulated phosphodiesterases mediate a variety of cellular effects. In addition to cGMP-dependent pathways, cGMP-independent regulation of many biological functions exists, including modification of proteins through direct chemical reactions. For example, S-nitrosylation of cysteine thiol residues by NO occurs independently of cGMP and it mediates several physiological functions (Stamler, *et al.*, 2001). For example, NO inhibits caspase 3 activity by S-nitrosylation of Cys163 residue thereby decreasing apoptosis of umbilical vein endothelial cells (Rossig, *et al.*, 1999).

1.6.1 NO, Cell Proliferation and Cancer

NO and its metabolites such as nitrate, nitrite, nitrosamines, peroxynitrite, and S-nitrosothiols play a variety of roles in promoting cytotoxic and genotoxic effects, including DNA and protein damage, loss of protein function, apoptosis, necrosis, gene mutation and inhibition of mitochondrial respiration (Lala and Chakraborty, 2001; Wink, *et al.*, 1998; Wink, *et al.*, 1998). Therefore, NO may participate in causation and progression of cancers. In fact, a large number of studies have associated NO with cell survival, progression, angiogenesis, and invasiveness (Fukumura, *et al.*, 2006).

Continuous exposure to high levels of NO that are generated by iNOS are believed to promote neoplastic transformation, which is an important initial step in cancer. NO can cause DNA damage by the generation of dinitrogen trioxide (N₂O₃) and

peroxynitrite (ONOO⁻). N₂O₃ nitrosates amines to form nitrosamines, and then alkylates DNA. Similarly, ONOO⁻ can oxidise and nitrate DNA, and may induce single strand breaks by attacking the sugar phosphate backbone. NO metabolites may also inhibit DNA repair enzymes, such as DNA ligase (Lala and Chakraborty, 2001; Wink, *et al.*, 1998; Xu, *et al.*, 2002), resulting in the accumulation of DNA damage. S-nitrosylation of caspases can produce apoptosis-resistant cells, and facilitate the accumulation of mutations and subsequent clonal selection (Lala and Chakraborty, 2001). Many studies have indicated that NO produced by iNOS can initiate and/or promote tumourigenesis (Crowell, *et al.*, 2003; Hofseth, *et al.*, 2003). For instance, mice with mutations in the genes of both adenomatous polyposis coli (*Apc*) and iNOS showed fewer polyps in the small and large intestines, as compared to the mice with mutation only in *Apc* (Ahn and Ohshima, 2001). In some experimental models, induction of iNOS in tumour cells led to the increase in tumour growth whereas, antisense iNOS decreased tumour growth (Ambs, *et al.*, 1998; Jenkins, *et al.*, 1995; Yamaguchi, *et al.*, 2002).

NO may also play a favourable role by being pro-apoptotic, protecting cells from cytotoxicity or by inhibiting cell proliferation (Heller, *et al.*, 1999; Wink, *et al.*, 1996). A study by Dong and his colleagues showed that endogenous NO could reduce the metastatic potential of metastatic melanoma, since transfection of iNOS to melanoma cells resulted in a dramatic decrease in metastasis (Dong, *et al.*, 1994). In another instance, NO was shown to reduce metastasis by inhibiting the adhesion of tumour cell to the venular side of the microcirculation (Kong, *et al.*, 1996). Another report suggests that NO produced by the hepatic endothelium prevented the metastasis of lymphoma cells (Rocha, *et al.*, 1995). Similarly, NO produced in the vasculature of the brain limited the

spread of colon cancer to the brain (Murata, *et al.*, 1997). The same group also demonstrated that NO, secreted by microglial cells, may suppress the spread of cancer to the brain (Murata, *et al.*, 1997). All of these studies when put together suggest that NO can either promote or suppress the growth of tumour cells. The tumour promoting or inhibiting ability of NO depends on a variety of factors, such as NO concentration, cell type and the local microenvironment.

1.7 Rationale

The upregulation of CPD by PRL, E2 and/or R1881 in breast and prostate cancer cell lines increases NO production, decreases cell apoptosis and increases viability (Abdelmagid and Too, 2008; Thomas, *et al.*, 2012). It is possible that PRL and R1881 activate CPD gene transcription through Stat5 and the liganded-AR, respectively, with Stat5 binding to the GAS motif, and the AR to the ARE(s) found in the CPD gene promoter. The potential binding sites for PRL-activated Stat5 and liganded AR are in close proximity in the CPD gene promoter, suggesting the possibility that Stat5 and the AR could act cooperatively. An understanding of CPD gene transcription would give insight into the convergence of hormonal action and interaction that lead to cell survival.

1.8 Hypothesis

In the presence of both PRL and R1881, the activated Stat5 and liganded AR bind to the putative GAS and ARE(s), respectively, in the CPD gene promoter. The two active transcription factors act cooperatively to enhance each other's binding to their DNA binding sites, to stimulate CPD gene transcription in breast cancer cells.

1.9 Objectives:

- i) To determine E2, PRL and androgen regulation of CPD gene expression using Western and qPCR analyses.
- ii) To determine hormonal regulation of CPD promoter activity using luciferase reporter assays.
- iii) To identify active transcription factor binding sites in the CPD gene promoter by gene mutation and CHIP assays.
- iv) To investigate possible cooperativity between Stat5 and AR in the activation of CPD gene promoter.

CHAPTER 2. MATERIAL AND METHODS

2.1 Antibodies

The concentrations and sources of primary antibodies used were: Custom-made, affinity-purified rabbit anti-CPD (1:500); rabbit anti-Stat5 (1:1000) and rabbit anti-pStat5 (1:1000) from Cell Signalling (Danvers, MA); mouse anti-androgen receptor (1:1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); rabbit anti-actin (1:5000) from Sigma-Aldrich Canada, Ltd. (Oakville, ON). The secondary antibodies used were goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate and goat anti-mouse IgG HRP conjugate (Santa Cruz Biotech. Inc.).

2.2 Cell culture

Human MCF-7 and T47D breast cancer cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂. MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), pH 7.4, containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), and supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 50 U/ml of streptomycin/penicillin. T47D cells were maintained in DMEM containing 10% FBS, and supplemented with 2 mM L-glutamine, 5 mM HEPES and 50 U/ml of streptomycin/penicillin. Cells were growth-arrested in medium containing 1% charcoal-stripped serum (CSSM) for 48 h prior to treatment with various hormones and inhibitors.

2.3 Transfection of Plasmid

MCF-7 cells in growth medium containing 10% FBS were seeded at a density of 4×10^5 cells per well in 6-well plates. After 24 h, the medium was removed. The cells were washed with phosphate-buffered saline (PBS), pH 7.4, and re-incubated in serum-free DMEM. Plasmids were prepared by incubating pGL3-CPD (500 ng) (gift from Dr. R. A Skidgel, University of Illinois), phRL-TK (5 ng; for normalizing transfection efficiency), with PLUS™ reagent (Invitrogen, Canada Inc., Burlington, ON, Canada) at room temperature for 15 min. In negative controls, pGL3-CPD was replaced by pGL3-Basic (500 ng). LipofectAMINE™ reagent in serum-free medium was mixed with the PLUS™ mixture, and incubated at room temperature for 15 min. The total transfection mixture was added to MCF-7 cells in serum-free DMEM. After 5 h, transfected cells were replaced with 1% CSSM to growth-arrest the cells. After 48 h in arresting medium, the quiescent cells were treated with PRL (10 ng/ml), 17β-estradiol (E2) (10 nM) or synthetic androgen R1881 (10 nM). Control cells were left untreated. Cells were harvested using Passive Lysis Buffer (Promega Corp., Madison, WI) following the manufacturer's instructions.

2.4 Transfection of Small Interfering Ribonucleic Acid (siRNA)

MCF-7 cells were seeded in a 6-well plate at 4×10^5 cells/well or 2×10^6 cells in a 10 cm dish. The cells were transfected with Silencer® Select pre-designed siRNA targeting human Stat5a (ID s13534) and non-targeting controls (siNT, Cat. # 4390843), both purchased from Ambion® (Life Technologies, Inc., Burlington, ON, Canada). A final concentration of 10 nM siRNA was used for transfections. Transfections were

performed using Lipofectamine™ 2000 (Life Technologies, Inc.) following manufacturer's instructions. Following transfection, the cells were growth arrested for 48 h at 37°C. Cells were then treated with hormones for 6 h or left untreated. Cell lysates prepared from cells with knocked down genes were used in luciferase reporter assays.

2.5 Preparation of total cell lysates

Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL/octylphenoxypolyethoxyethanol (Sigma-Aldrich), 1 mM phenylmethylsulphonyl fluoride (PMSF), containing freshly prepared protease inhibitor cocktail P8340 (Sigma-Aldrich)). Cells were disrupted by passage through a 21-gauge needle and fresh PMSF (10 µg/ml) was again added. Cell lysates were centrifuged at $13,000 \times g$ for 20 min at 4°C to remove cell debris. The supernatant was collected (total cell lysate), and used for Western analysis or frozen at -20°C until further analysis.

2.6 Protein assay

Protein concentrations of total cell lysates were determined using the Bradford assay. Samples were prepared by diluting 50 µl of the total cell lysate in 150 µl of H₂O. A standard curve was prepared using bovine serum albumin (BIO-RAD) of known concentrations (i.e., 0.2, 0.4, 0.6, 0.8 µg/µl). Five millilitres of dye reagent concentrate (BIO-RAD) was added to 95 µl of the standards and samples. Absorbance was measured at 595 nm using Eppendorf® Biophotometer.

2.6 Sodium dodecylsulphate and polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using protein samples that were diluted with 50% volume of 3X SDS-PAGE loading buffer (188 mM Tris Chloride of pH-6.8, 3% SDS, 30% glycerol, 0.01% bromophenol blue, 15% β -mercaptoethanol). Protein samples (30-50 μ g/lane) in loading buffer were incubated for 5 min at 95°C, and then loaded onto 7 or 10% SDS-polyacrylamide gels. The resolving gel components were: 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS), and 0.05% tetramethylethylenediamine (TEMED). Stacking gel (4%) components were: 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% APS, and 0.05% TEMED. Protein samples were resolved at 200 V in SDS-PAGE running buffer (0.02 M Tris-HCL, pH 8.3, 0.2 M glycine and 0.1% SDS).

2.7 Western analysis

Following SDS-PAGE, resolved proteins were transferred onto BiotraceTM NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida) in Western transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol), at 100 V for 2 h at 4°C or 30 V overnight (O/N) at 4°C. Nitrocellulose membranes were blocked in 10% skim milk powder (w/v) in Tris-buffered saline-Tween 20 solution (TBST; 0.02 M Tris-HCl, pH 7.6, 0.2 M NaCl, 0.05% Tween 20) for 1 h at room temperature or O/N at 4°C. The nitrocellulose blots were then incubated with primary antibody prepared in 10% skim milk in 0.05% TBST for 2 h at room temperature or O/N at 4°C. The blots were washed 3 times with TBST for 15 min each time. The blots were then incubated with secondary antibody prepared in 10% skim milk in TBST for 1 h. The same washing cycle was

repeated. Immunoreactive signals were detected using BIO-RAD Immun-StarTM WesternCTM Kit by spreading 1:1 mixture of reagents (Peroxide: luminol solution) onto the blot for 5 min prior to detection by chemiluminescence.

2.8 Luciferase assays

Reporter assays were performed using Dual Luciferase[®] Assay kit (Promega Corp., Madison, WI) and the activity of the promoter was analysed using Luminoskan Ascent Luminometer (Thermo Labsystems, Franklin, MA). Cell lysates (20 µl/well) were added to a 96-well plate. A volume of 100 µl each of LAR II and Stop & Glow[®] Reagent was added to each well. The luminescence intensity of firefly luciferase normalized to that of Renilla luciferase was measured on Luminoskan Ascent. All experiments were carried out in duplicate wells and repeated three times. The following formula was used to calculate relative CPD promoter activity; $\text{Relative CPD promoter activity} = \frac{\text{Luminescence intensity of test}}{\text{Luminescence intensity of controls}}$.

2.9 Reverse transcription and polymerase-chain reaction (RT-PCR)

RNA was isolated from MCF-7 cells using GenElute Mammalian Total RNA miniprep kit following the manufacturer's instructions (Sigma-Aldrich). Total RNA (1 µg) was incubated with 1 µl of DNaseI and 1 µl of 10X reaction buffer (Fermentas, Burlington, Ontario) in a total volume of 10 µl for 30 min at 37°C. Then, 1 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) was added to stop the DNase reaction, and incubated at 70°C for 10 min. RT of RNA (10 µl) was performed in a 25 µl reaction mixture containing M-MuLV reverse transcriptase (100 U; Promega), 40 pM of random hexamer pd(N)₆, 200 µM of deoxynucleotide triphosphates (dNTPs), and 1.6 U RNase

inhibitor. A 3- μ l aliquot of RT reaction was used for PCR containing 12.5 μ l of GoTaqTM Green Master Mix (Promega Corp., Madison, WI), 0.5 μ l of forward and reverse primers in a total volume of 25 μ l. The following primer sets were used: human CPD, 5'-ATG-GCA-GGG-GTA-TAT-TAA-ATG-CAA-3' and 5'-GGA-TAC-CAG-CAA-CAA-AAC-GAA-TCT-3' (576 bp); human actin, 5'-AAA-CTG-GAA-CGG-TGA-AGG-TG-3' and 5'-AGA-GAA-GTG-GGG-TGG-CTT-TT-3' (172 bp); human Stat5a, 5'-ACA-TTT-GAG-GAG-CTG-CGA-CT-3' AND 5'-CCT-CCA-GAG-ACA-CCT-GCT-TC-3'.

2.10 Quantitative polymerase chain reaction (qPCR)

Preparation and RT of total RNA, isolated from quiescent cells treated with various doses of PRL or R1881, was performed as for RT-PCR described above. A 3- μ l aliquot of RT reaction was combined with GoTaq qPCR master mix (Promega) or Brilliant II SYBR Green qPCR Master Mix, diluted ROX reference dye (Agilent Technologies Canada Inc., Mississauga, ON, Canada) and primers, according to the manufacturer's instruction. The following sets of primers were used: hCPD, 5'-AAC-ACC-ACC-GAC-GTG-TAC-CT-3' and 5'-GTG-CTA-AAC-TGG-TCG-GGA-AA-3' (164-bp product). The primers for human actin were the same as described for RT-PCR. Reaction mixes were amplified through 40 cycles in a Stratagene MX3000P thermal cycler. Target gene expression was normalized to actin levels in respective samples as an internal standard and was determined using the comparative cycle threshold (Ct) method. Briefly, Δ Ct was calculated for each treatment group by subtracting the Ct for actin from the Ct for CPD. The $\Delta\Delta$ Ct for each group was calculated by subtracting the Δ Ct for the calibrator (0 timepoint) from the Δ Ct for the sample. Therefore, $\Delta\Delta$ Ct = (Ct_{CPD} –

$C_{\text{actin}})_{\text{sample}} - (C_{\text{CPD}} - C_{\text{actin}})_{0 \text{ timepoint}}$. The normalized expression in each sample equals $2^{-\Delta\Delta C_t}$. This value was used to compare the expression levels.

2.11 Immunoprecipitation

For immunoprecipitation of Stat5 and AR, total cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was then precleared with IgG and A/G PLUS agarose (Santa Cruz), and recollected at 300 rpm for 5 min. Precleared lysates were then incubated with 1 µg of Stat5 and AR antibodies for 1 h, and then with 40 µl of A/G agarose at 4°C. Agarose beads were washed three times with PBS and then centrifuged at 3000 x g for 5 min at 4°C. The washed beads were suspended in 3X sample buffer and used in Western analyses.

2.12 Chromatin Immunoprecipitation Assay

MCF-7 cells were seeded at 2×10^6 cells in a 10 cm dish. The cells were grown in regular growth medium (for analyses of RNA Pol II and IgG) or serum starved and then treated with 10 nM PRL or 10 nM R1881 (for analyses of Stat5 and AR, respectively). Cells were trypsinized, washed with PBS and crosslinked with 37% formaldehyde (final concentration of 1%). Reactions were terminated by adding 1 ml of 0.125 M glycine and subjected to centrifugation for 5 min at 180 x g. Pelleted cells were then washed three times with ice-cold PBS. Cell nuclei were prepared by resuspending the pellet in nuclei preparation buffer containing 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM ethylene glycol-bis *N,N,N',N'*-tetra-acetic acid (EGTA), 15 mM Tris-Cl pH 7.5 and protease inhibitor cocktail (PIC, Sigma Aldrich). Cell suspensions were incubated on ice for 15 min, vortexed vigorously for 10 sec and then

centrifuged at 180 x g for 5 min. The nuclear pellet was resuspended in micrococcal nuclease (MNase) digestion buffer and incubated for 8 mins at 37°C. MNase (New England Biolabs[®] Ltd. Whitby, ON, Canada) was added to the nuclear suspension at a final concentration of 2 U/ml for 20 mins at 37°C to obtain the DNA fragments of 100-400 bp. MNase reactions were terminated by adding 100 ul of 0.5 M EDTA to the digested DNA. Cell debris was pelleted by centrifuging at 14,000 rpm for 15 mins at 4°C. The clarified supernatant was then used for immunoprecipitation with anti-Stat5, anti-AR, anti-RNA Pol II or IgG, crosslink reversal, and purification of DNA following manufacturer's instructions (Imprint[®] Chromatin Immunoprecipitation Kit, Sigma Aldrich). Specific primer pairs were used to amplify the promoter region GAS and ARE-1 of CPD gene promoter. The primers used for GAS region amplification were 5'-TGT-GCT-CCC-TGA-AGA-CTG-GC-3' and 5'-CCA-TGG-AGG-ATG-GGA-AGG-A-3' or 5' -TGG-CCT-TCC-AGA-TCA-AAT-GT-3' and 5' - GGT-GAG-GGA-AAT-ATC-TGA-A-3' for ARE-1 region or 5'-TAG-ACT-AGA-TCA-AAT-GGG-GC-3' and 5'-ATC-TGG-AGA-CAA-AGA-GGA-TTG-3' for ARE-2 region. The primers of a non-related gene, GAPDH, were used as a negative control.

2.13 Generation of CPD constructs

A 2.0-kbp CPD promoter construct, a kind gift from Dr. R.A. Skidgel, was used as a template to mutate or delete putative GAS and AREs. Mutagenesis was performed using the Phusion Site Directed Mutagenesis Kit (New England Biolabs[®] Ltd. Whitby, ON, Canada). PCR was performed using Phusion polymerase and phosphorylated primers. Following the reaction, digestion was performed with DpnI to chew up the 2.-kbp CPD template. The DpnI digested PCR product was transformed into chemically

competent DH5 α cells (*Escherichia Coli*). The following mutants were generated, and their sequence was verified by DNA sequencing (Genewiz Inc., South Plainfield, NJ): Δ ARE2,3-CPD (deletion of two putative AREs), Δ ARE2,3,GAS-CPD (deletion of GAS in addition to two putative AREs), and Δ GAS-CPD (substitution mutation of putative GAS element). The primers used in the mutagenesis were; Δ ARE2,3-CPD, 5'-AGT-CTT-AGG-CCT-CCC-ATT-CAA-TTT-TCC-3' and 5'-AGA-GAG-ACC-GCC-CAC-CAC-CT-3'; Δ ARE2,3,GAS-CPD, 5'-CAA-GGC-ATT-TGT-TCT-CAA-ATC-CTG-C-3' and 5'-AGA-GAG-ACC-GCC-CAC-CAC-CT-3'; Δ GAS-CPD, 5' -CAA-GGC-ATT-TGT-TCT-CAA-ATC-CTG-C-3' and 5'-TGT-TTT-TTT-TTT-TGC-ATG-CAG-GGT-C-3'.

2.14 Statistical Analysis:

Statistical analyses were performed using Graphpad prism, and the results were expressed as mean \pm SEM ($n \geq 3$) unless otherwise stated in the figure legends. ANOVA was followed by Benferroni's multiple comparison tests, for comparing mean value. A *P* value of ≤ 0.05 was considered significant.

CHAPTER 3. RESULTS

3.1 CPD mRNA expression is upregulated by PRL, E2, and R1881 treatment

We have previously reported that the treatment of quiescent MCF-7 cells with PRL and 17- β -estradiol (E2) stimulated CPD mRNA production (Abdelmagid and Too, 2008). My study focussed on the regulation of CPD gene expression and transcription by PRL, E2, and R1881 (synthetic non-aromatizable androgen). In the first instance, time- and dose- dependent studies were performed. MCF-7 cells were growth arrested in 1% CSSM for 48 h prior to hormonal treatment. E2 (10 nM) and PRL (10 ng/ml) stimulated CPD mRNA levels by greater than 4 fold (Figure 5A, B). Similarly, R1881 (10 nM) upregulated CPD mRNA levels by up to 6 fold at 6 h of treatment (Figure 5C).

3.2 CPD protein levels is upregulated by PRL, E2, and R1881 treatment

The effects of E2, PRL, and R1881 on CPD protein levels were next determined, using Western analysis. The treatment of quiescent MCF-7 and T47D cells with E2 and PRL stimulated CPD protein levels at 6 h (Figure 6A, B). The present study also showed that R1881 upregulated CPD protein levels in a time- dependent manner (Figure 6C).

3.3 Stimulation of CPD mRNA by PRL and R1881 was suppressed by actinomycin D

Treatment of MCF-7 cells with PRL, E2 or R1881 increased CPD levels in a time- and dose-dependent manner (Figure 5 and 6). To determine the mechanism by which CPD mRNA expression was regulated by these hormones, MCF-7 cells were pretreated with or without actinomycin D (2.5 μ g/ml) for 30 min before treatment with hormones for 6 h. In the presence of actinomycin D, PRL- and R1881-upregulated CPD

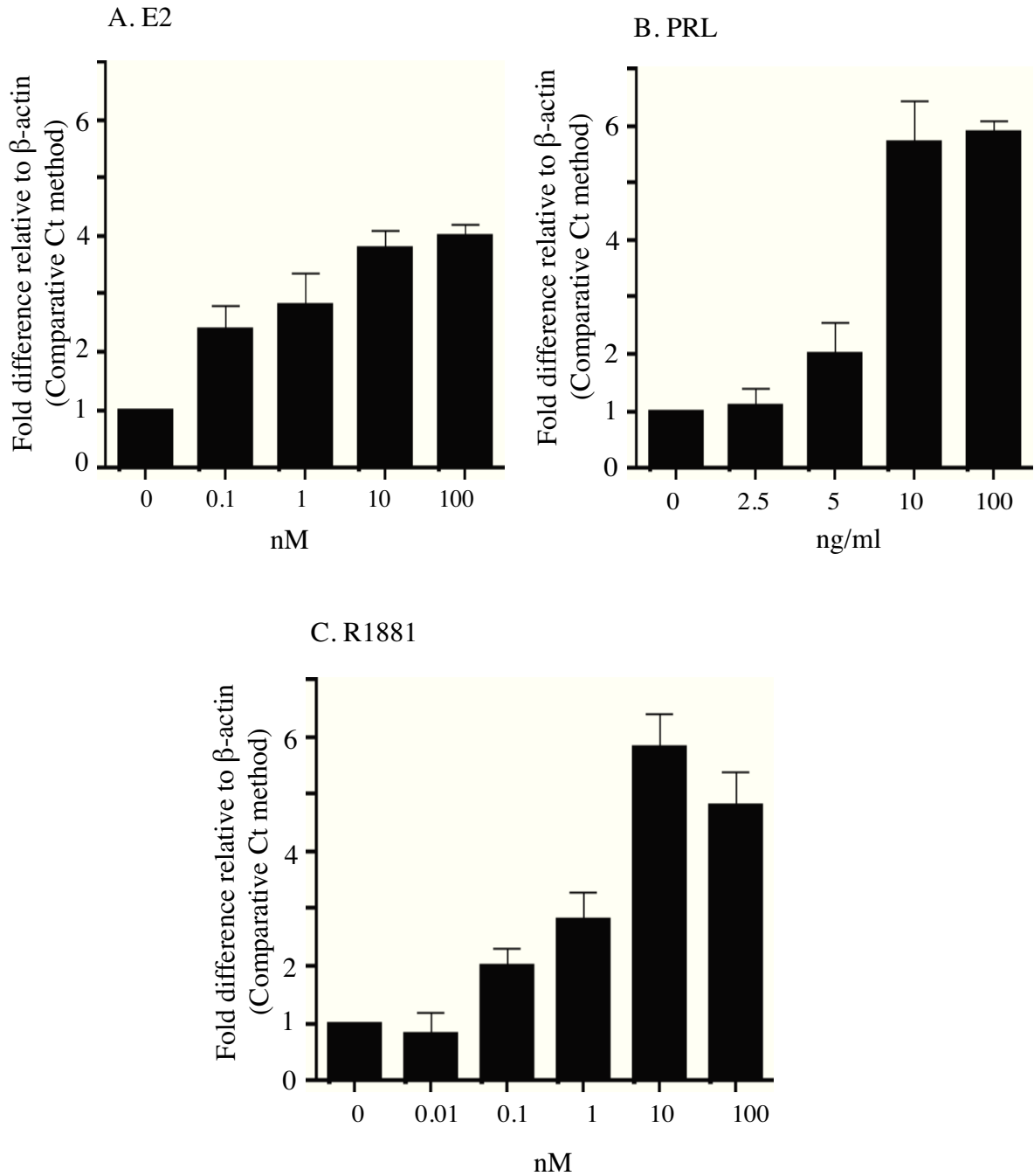


Figure 5. Dose-dependent stimulation of CPD mRNA levels. MCF-7 cells were arrested in 1% charcoal-stripped serum containing medium (CSSM) for 48 h prior to treatment with increasing doses of A) E2, B) PRL, or C) R1881 for 6 h. Total RNA was extracted for qPCR analysis. The CPD/ β -actin ratio was determined by the $2^{-\Delta\Delta Ct}$ method, which showed ≥ 4 -fold induction with 10 ng/ml PRL, 10 nM E2 or 10 nM R1881. Mean \pm SEM, n=3.

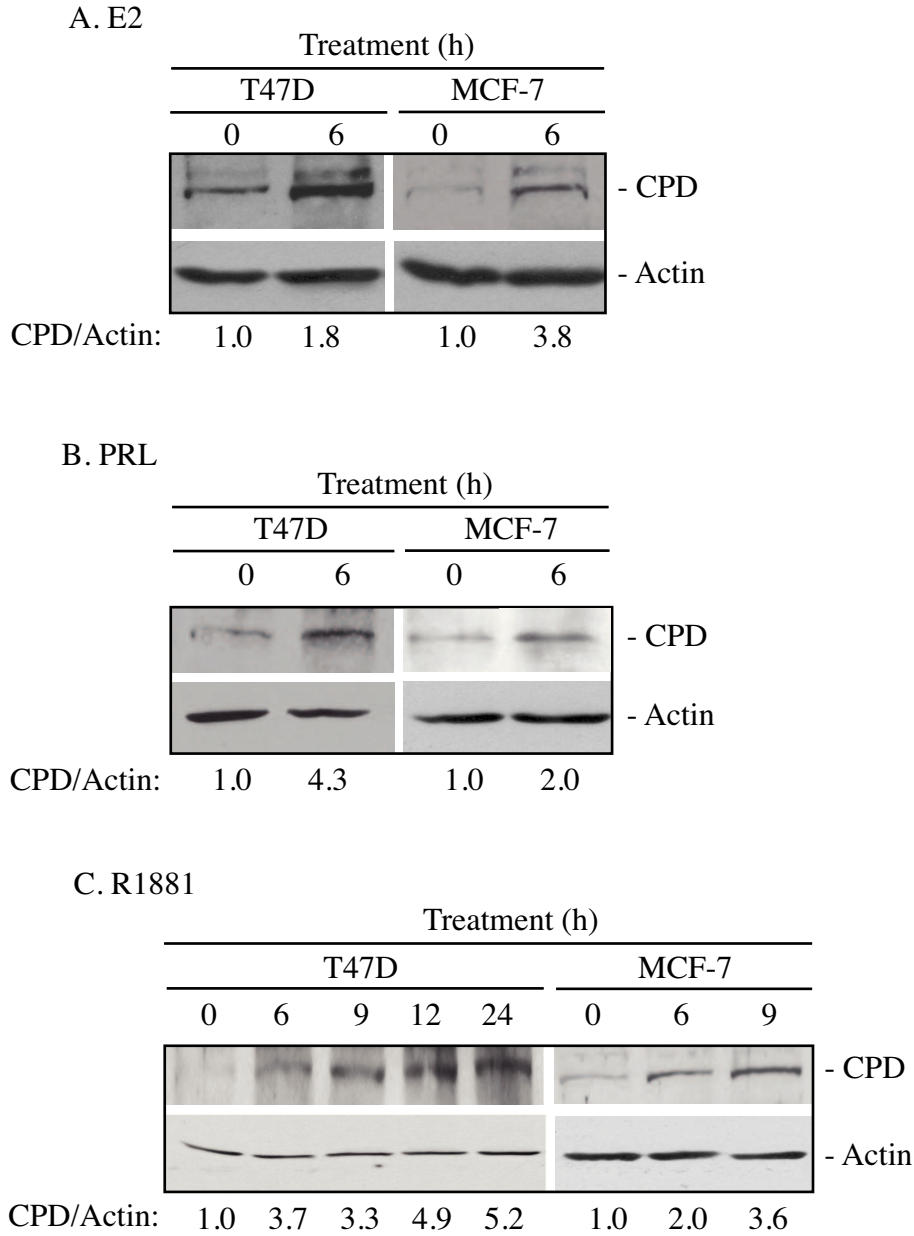


Figure 6. Hormonal treatment stimulates CPD protein levels in MCF-7 and T47D cells. MCF-7 and T47D cells were made quiescent in 1% CSSM for 48 h prior to the treatment with A) E2 (10 nM), B) PRL (10 ng/ml), or R1881 (10 nM) for 6 h. Total cell lysates were prepared for SDS-PAGE and Western analysis as described in Materials and Methods. For SDS-PAGE, 20-40 μ g of protein was loaded in each lane. The 180-kD immunoreactive CPD band was detected by chemiluminescence. β -actin was used as a loading control. Representative of three independent experiments.

mRNA levels were markedly reduced as compared to the cells without actinomycin D (Figure 7), suggesting transcriptional regulation by these two hormones. Surprisingly, actinomycin D did not abolish E2-stimulated CPD mRNA levels, suggesting that E2 upregulation of CPD mRNA was not regulated at the transcriptional level. Actinomycin D treatment alone did not change the viability of MCF-7 cells.

3.4 CPD mRNA induction by PRL, E2 and R1881 does not require synthesis of new proteins

To determine whether the induction of CPD mRNA levels requires *de novo* protein synthesis, I evaluated the effect of a protein synthesis inhibitor, cycloheximide on CPD mRNA expression in MCF-7 cells challenged with hormone. MCF-7 cells were made quiescent for 48 h, and then treated with PRL or E2 or R1881 for 6 h, with or without cycloheximide (25 µg/ml). QPCR analysis showed that cycloheximide had no effect on the hormonal upregulation of CPD mRNA levels (Figure 8). This suggests that hormonal upregulation of the CPD mRNA was independent of *de novo* protein synthesis. However, this does not exclude factors that modulate gene transcription or contribute to the stability of the transcript.

3.5 CPD expression is regulated by mRNA stability

CPD mRNA levels is upregulated by E2 in MCF-7 cells (Figure 5B). However, unlike PRL and R1881, the upregulation of CPD mRNA levels by E2 was not abolished by transcriptional inhibitor actinomycin D (Figure 7), suggesting that the E2 did not affect gene transcription. Therefore, I investigated whether E2 induction of CPD mRNA

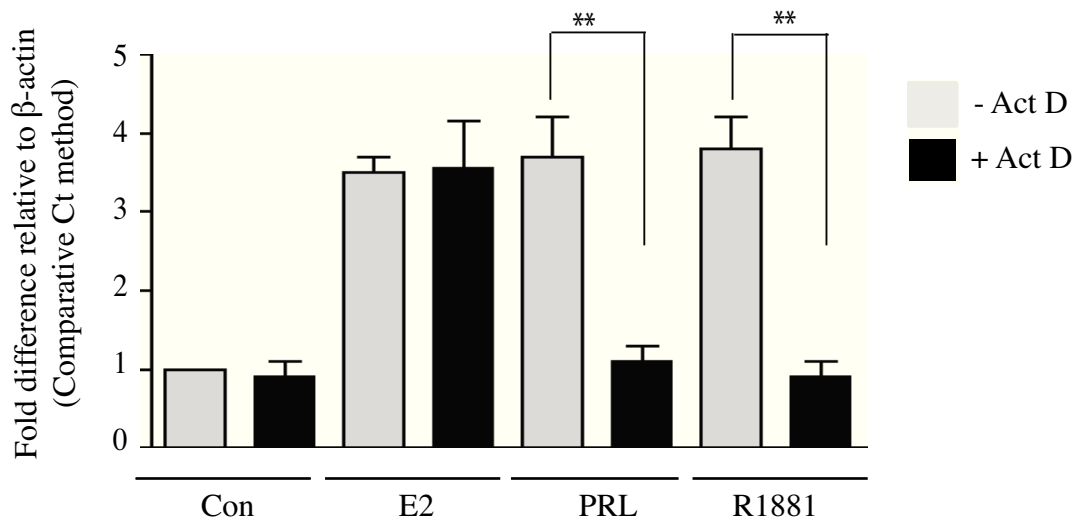


Figure 7. Effects of actinomycin D in CPD mRNA expression. MCF-7 cells were growth arrested in 1% CSSM for 48 h. The cells were treated with or without Act D (2.5 $\mu\text{g/ml}$) for 30 min, followed by PRL (10 ng/ml), E2 (10 nM) or R1881 (10 nM) for 6 h. Controls (Con) were not treated with hormones. Total RNA was extracted for qPCR analysis. The relative CPD mRNA levels were calculated using β -actin as a reference and the control mRNA levels were expressed as 1. Mean \pm SEM, n=3-4. **p<0.001.

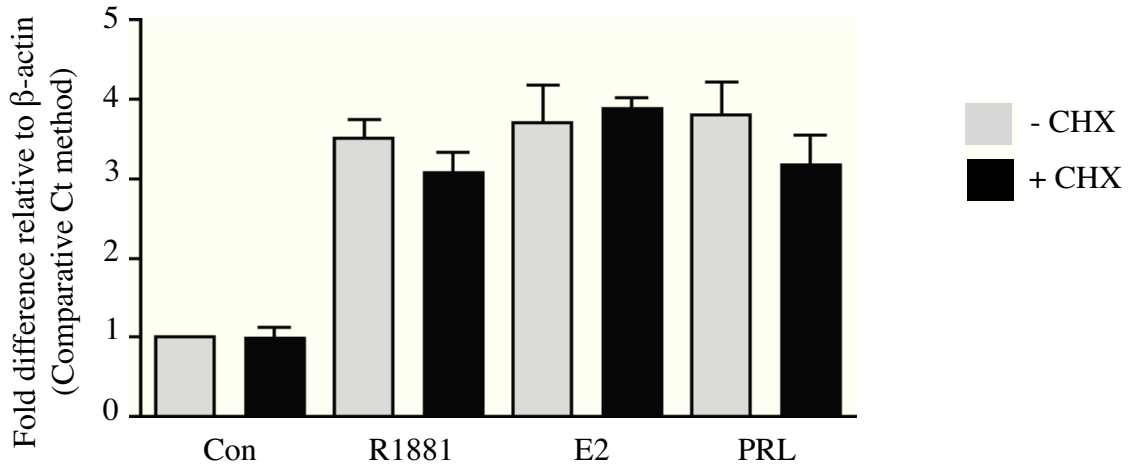


Figure 8. CPD mRNA induction by PRL, E2 and R1881 does not require synthesis of new proteins. MCF-7 cells were growth arrested in 1% CSSM for 48 h prior to the treatment with or without CHX (10 μ g/ml) for 30 min, followed by E2 (10 nM), PRL (10 ng/ml), or R1881 (10 nM) for another 6 h. Controls (Con) were not treated with hormones. Total RNA was isolated after 6 h of treatment. For RT-qPCR analyses, the relative mRNA levels were calculated using β -actin as an internal standard. Control mRNA levels were expressed as 1. Mean \pm SEM, n=3-4.

expression was through other mechanisms, such as stabilization of the transcript. Therefore, MCF-7 cells were growth arrested for 48 h and total mRNA was collected from a set of cells before any treatment (time zero). Another set of cells were then either treated with E2 (10 nM) or actinomycin D alone, or pre-treated with actinomycin D for 30 min before incubating the cells with E2 for 8 h. Total RNA was collected at different times and processed for RT-qPCR analysis. In the cells treated with actinomycin D alone, CPD mRNA degradation was rapid (Figure 9), indicating that the CPD transcript had a short half-life. However, in cells treated with E2 + actinomycin D, or with E2 alone, CPD mRNA levels were maintained higher and for a much longer duration, suggesting that E2 increased the stability of CPD mRNA. Statistical analysis showed that the E2 alone or E2+actinomycin D profile were not significantly different from each other, but each was significantly higher than the actinomycin D treatment alone at 4 h ($P < 0.01$, $n=3$ for E2 + actinomycin D *versus* actinomycin D; $P < 0.001$, $n=3$ for E2 *versus* actinomycin D) and 8 h ($P < 0.001$, $n=3$ for both E2 + actinomycin D *versus* actinomycin D and E2 *versus* actinomycin D). These results support the idea that E2 increases the stability of the CPD mRNA, thereby increasing its half-life in MCF-7 cells.

3.6 The three CPD luciferase promoter constructs (0.7-, 2.0- and 7-kbp) are robustly active and the 2.0-kbp construct is stimulated by PRL and R1881

CPD gene promoter activity was next studied to unravel the pathways involved in the stimulation of the CPD gene. We have three CPD promoter constructs of different sizes, that is, 0.7-, 2.0- and 7-kbp upstream from the start site. We have identified one consensus γ -interferon activating sequences (GAS) and four putative AREs in the 7.0-

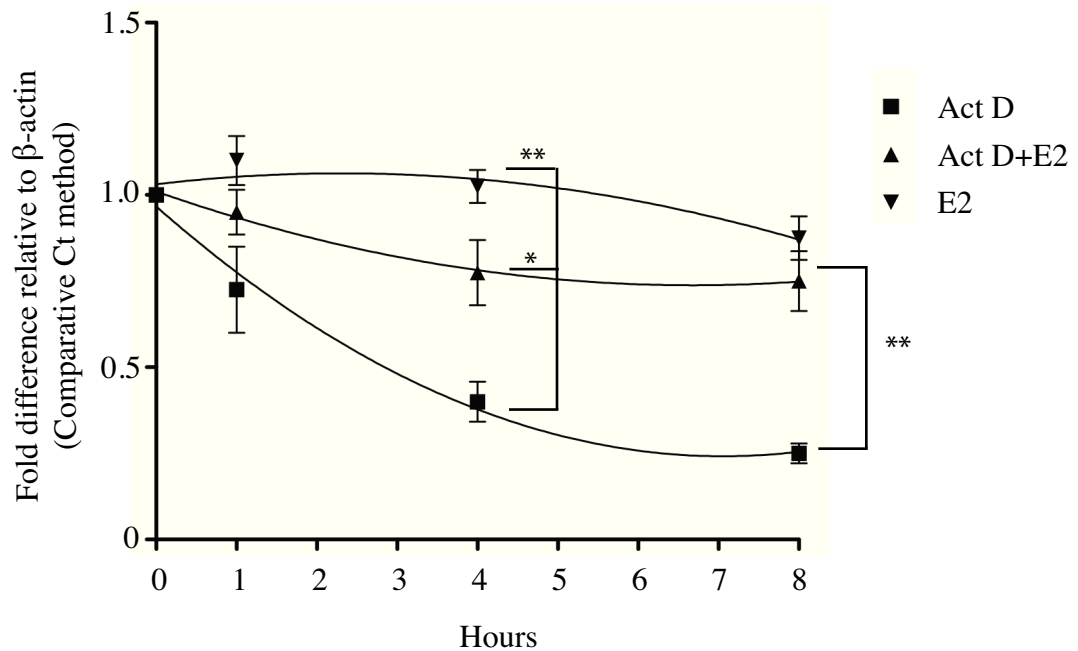
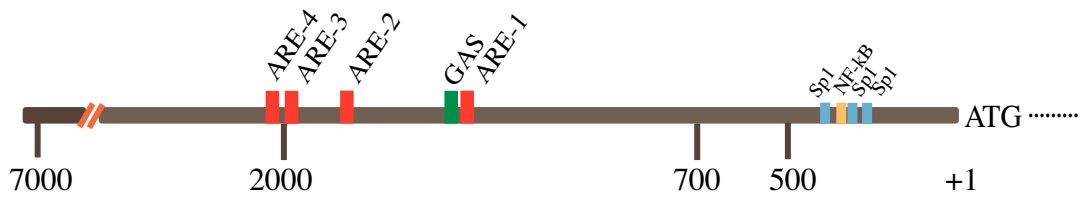


Figure 9. E2 increases CPD mRNA stability. MCF-7 Cells were growth arrested with 1% CSSM for 48 h. The cells were treated with actinomycin D (Act. D) (2.5 ug/ml) alone or pretreated with Act. D or vehicle (ethanol) for 30 min prior to the addition of E2 (10 nM) as shown in the figure. Total RNA was isolated at the indicated times. The relative mRNA levels were calculated using β -actin as a reference. The relative amounts of CPD mRNA at time zero was expressed as 1. Mean \pm SEM, n=3. *P<0.01 **P<0.001 when compared to Act D treatment.

kbp construct (Figure 10). The 2.0-kbp-promoter construct contains the GAS and three of the four AREs, whereas the 0.7-kbp promoter construct does not have any of them (Figure 10). MCF-7 cells were transfected with each of these luciferase CPD-promoter constructs (0.7-, 2.0- and 7.0-kbp). After 5 h of transfection, the cells were cultured in regular growth medium for 48 h before performing luciferase reporter assays. All the promoter constructs were robustly active as compared to the empty vector, pGL3-Basic. This is in agreement with a previous report characterizing these promoter constructs in HEPG2 and Chinese hamster ovary (CHO) cells (Timblin, *et al.*, 2002) (Figure 11A).

To determine whether the hormones stimulate the CPD promoter constructs, each of the promoter constructs was transfected into MCF-7 cells. The cells were serum starved for 48 h and then treated with PRL, E2 or R1881 for 6 h. The 0.7-kbp CPD promoter construct did not respond to the hormones, possibly due to the absence of hormone response elements, namely ARE, GAS and ERE (Figure 11B). The activity of 2.0-kbp CPD promoter construct was increased by treatment with PRL or R1881 at 6 h, suggesting that PRL-activated Stat5 and the liganded androgen receptor might bind to the GAS and ARE/AREs, respectively. However, unlike PRL and R1881, E2 failed to stimulate CPD promoter (2.0-kbp) activity (Figure 11B), and this is consistent with the fact that E2 upregulation of CPD mRNA levels was not dependent on modulating CPD gene transcription. Surprisingly, the 7.0-kbp CPD promoter construct, which has all the response elements contained in the 2.0-kbp construct, failed to respond to treatment with PRL, E2 or R1881 (Figure 11B). This observation could be due to fact that, when large promoter constructs are cloned into a vector, they lack the appropriate *in vivo* microenvironment for gene transcription. Taking these results together, I concluded



Consensus GAS	5' -TTCnnnGAA-3'	
CPD GAS	5' -TTCccaGAA-3'	-1454
Consensus ARE	5' -GGTACAnnnTGTTCT-3'	
CPD ARE-1	5' - CAAGGC attTGTTCT-3'	-1443
ARE-2	5' - ACCTAA accTGTGCT-3'	-1848
ARE-3	5' - TGTACA gag TGTGG -3'	-1994
ARE-4	5' - TGTAAT agtTGAGCA-3'	-2036
Probasin ARE	5' - AGTACT cca AGAACC -3'	-136
PSA ARE	5' - AGCACT tgcTGTTCT-3'	-539
hPAR1 ARE	5' - CAACTT ctaTG TACA -3'	-539

Figure 10. The CPD promoter construct (7.0-kbp) contains one putative GAS and several putative androgen response elements (non-consensus). Transcription start site ATG is indicated as +1. Binding sites for Sp1 and NF-kB protein are present ~300 bp upstream of the start site. Our identified 5'-position of the putative androgen response elements (AREs) (1- 3) and γ -interferon activated sequence (GAS) in the CPD promoter are indicated. Active but non-consensus AREs present in probasin and prostate specific antigen (PSA) promoter are also shown. Non consensus nucleotides are shown in bold letters.

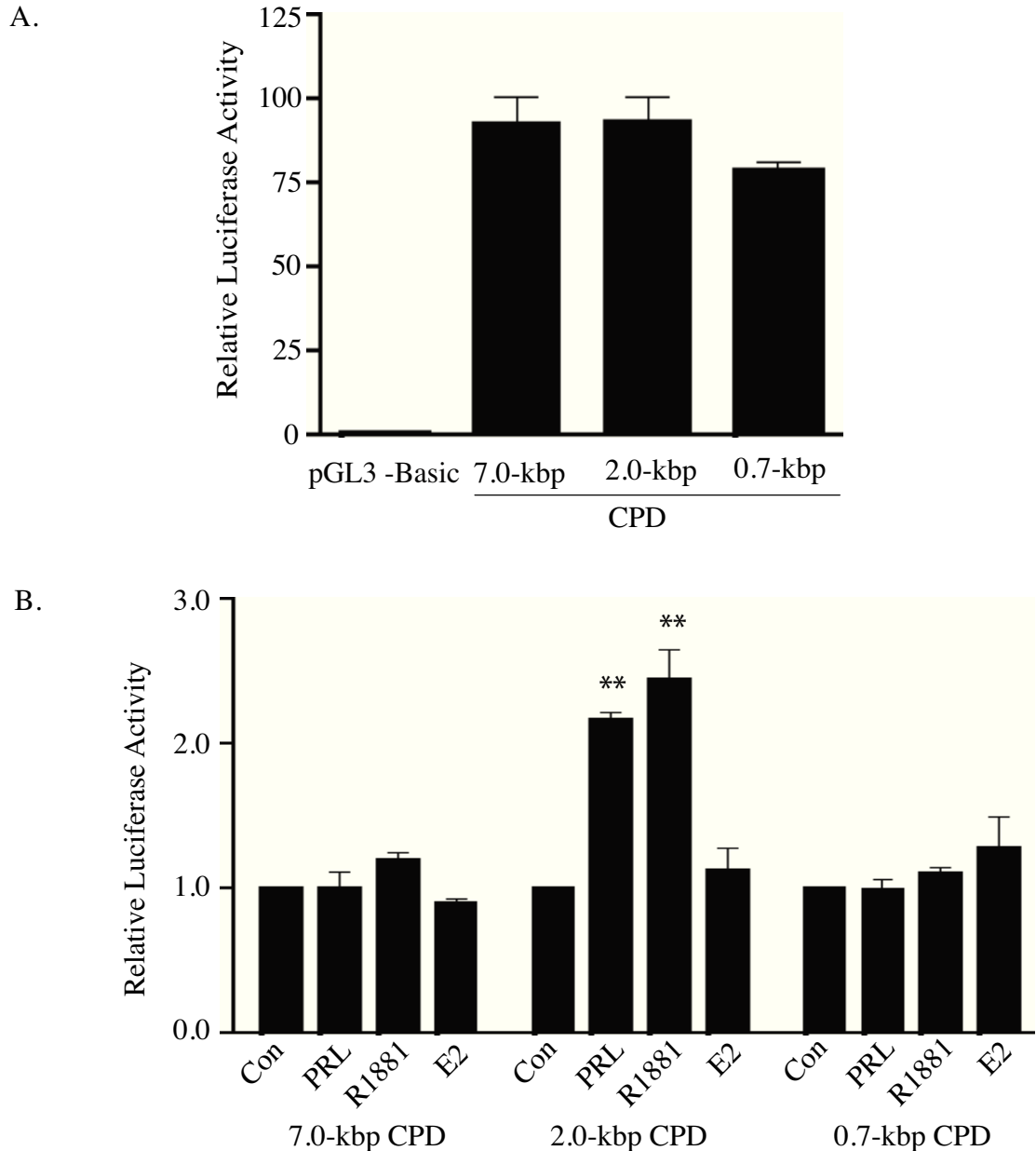


Figure 11. PRL and R1881 stimulate 2.0-kbp CPD promoter construct. MCF-7 cells were transfected with the CPD promoter-luciferase constructs (7.0- 2.0- or 0.7-kbp), empty vector PGL3-Basic (encoding firefly luciferase) or phRL-TK (encoding Renilla luciferase for normalization). A, The cells were cultured in complete growth medium containing 10% FBS for 48 h to determine promoter activity. B, After transfection, cells were growth arrested for 48 h in 1% CSSM and then treated with PRL (10 ng/ml), R1881 (10 nM) or E2 (10 nM) for 6 h. Cell lysates were used to determine luciferase reporter activity. Relative luciferase activity is presented as fold induction above baseline control which was arbitrarily set at 1. Three independent experiments were performed each in duplicate. Mean \pm SEM, n=3-9. ** P<0.001 compared to controls of the same promoter construct.

that the GAS and at least one of the AREs in the 2.0-kbp CPD promoter construct were active.

3.7 Deletion of two distal ARE-2 and ARE-3 from 2.0-kbp CPD promoter does not suppress PRL and R1881 stimulation of CPD promoter activity

Several new promoter constructs were generated from the 2.0-kbp construct by deletion or substitution mutations. The Δ ARE2,3-CPD was generated by the deletion of ARE-2 and ARE-3. The Δ ARE2,3-GAS-CPD was generated by the deletion of GAS from the Δ ARE2,3-CPD. The Δ GAS-CPD was generated by mutating GAS from the 2.0-kbp construct. All of the newly generated promoter constructs were robustly active in MCF-7 cells as determined by luciferase reporter assay (Figure 12A).

PRL and R1881 stimulated Δ ARE2,3-CPD promoter activity despite the deletion of two distal AREs, and the mutated construct responded in a magnitude similar to that of the full length 2.0-kbp CPD promoter (Figure 12B). These findings suggest that ARE-2 and ARE-3 (at -1443) were not involved in PRL or R1881 stimulation of CPD gene transcription.

3.8 PRL and R1881 failed to stimulate CPD promoter with mutated GAS

Since the stimulation of 2.0-kbp-promoter construct was not affected by the deletion of ARE 2 and ARE 3, we mutated the GAS to confirm that PRL stimulation of CPD involved this particular motif. As indicated, the two new promoter constructs Δ ARE2,3-GAS-CPD and Δ GAS-CPD were active (Figure 12A). As expected, PRL failed to stimulate the two promoter constructs that did not contain GAS (Δ ARE2,3-GAS-CPD and Δ GAS-CPD construct), suggesting that the PRL-stimulated Stat5 bound to this

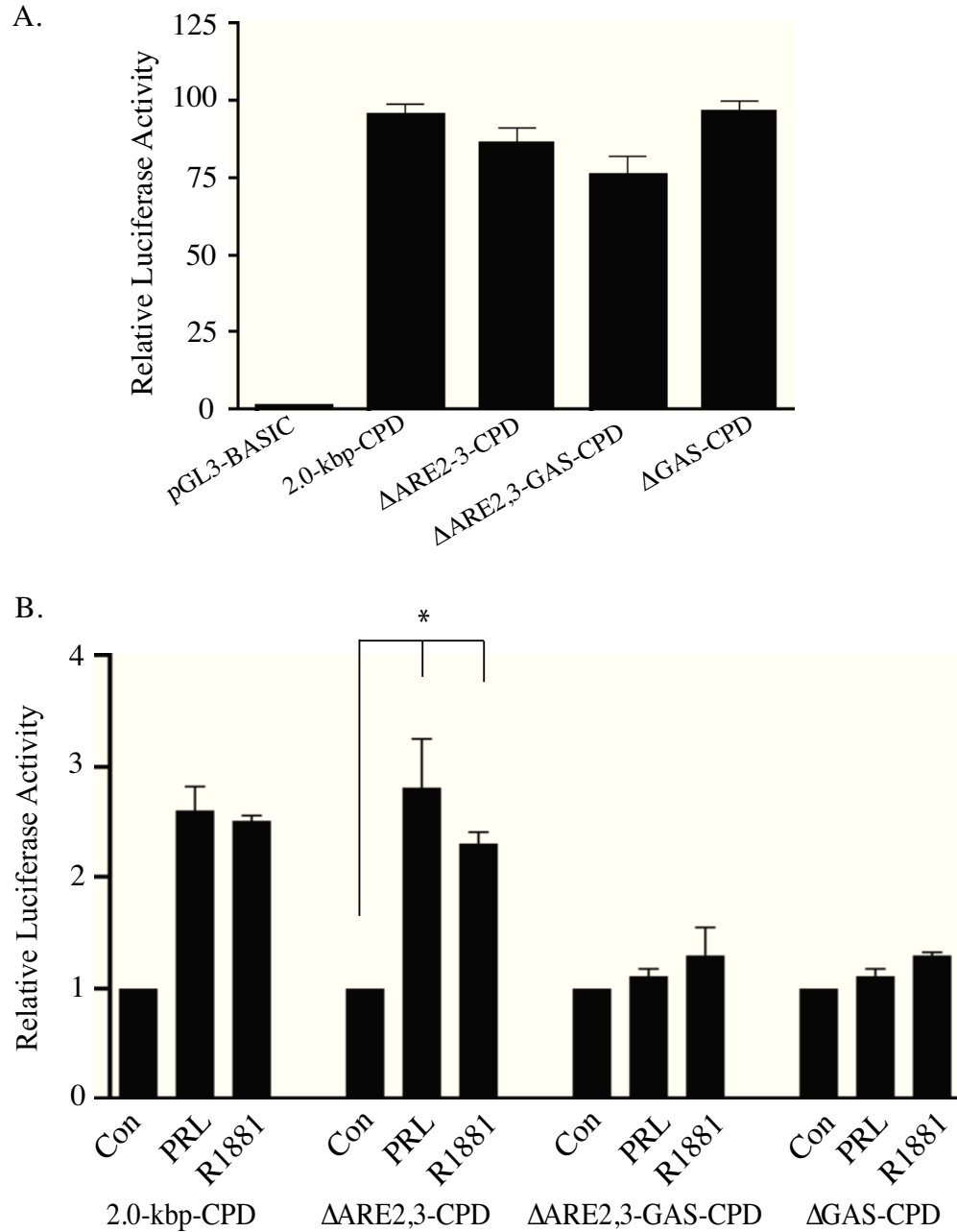


Figure 12. Mutation of GAS in the 2.0-kbp CPD promoter suppresses the stimulatory effects of PRL and R1881. MCF-7 cells were transfected with empty vector PGL3-Basic or CPD promoter-luciferase constructs (2.0-kbp, Δ ARE2-3, Δ ARE2,3-GAS, Δ GAS), and phRL-TK. After transfection, A, the cells were cultured in complete growth medium containing 10% FBS for 48 h. B, the cells were growth arrested for 48 h and were then treated with PRL (10 ng/ml) or R1881 (10 nM) for 6 h. Controls were left untreated. Cell lysates were used to measure luciferase reporter activity. Relative luciferase activity is presented as fold induction above baseline control which was arbitrarily set at 1. Three independent experiments were performed each in duplicate. Mean \pm SEM, n=3-5.* P<0.05.

particular GAS to activate gene transcription (Figure 12B). Surprisingly, although the R1881-bound AR does not bind to GAS, treatment with R1881 did not stimulate the Δ ARE2,3-GAS and Δ GAS-CPD promoters (Figure 12B). There is a possibility that the close proximity of the GAS and ARE-1 could allow cooperation between AR and Stat5 to stimulate CPD promoter activity. Therefore, experiments were conducted to determine whether the treatment with PRL + R1881 together would be necessary to stimulate Δ GAS-CPD promoter.

3.9 Sub-optimal doses of PRL in combination with R1881 stimulated Δ GAS promoter construct's activity

MCF-7 cells were treated with suboptimal doses of PRL (0.1 nM) or R1881 (0.1 nM). Alone, neither hormone stimulated the activity of the Δ GAS-CPD and the 2.0-kbp CPD construct (Figure 13B). However, when suboptimal doses of PRL and R1881 were given in combination, there was stimulation of the Δ GAS-CPD promoter constructs, although not to the level of the intact 2.0-kbp construct (Figure 13B).

To determine whether alterations in Stat5 levels would overcome the deletion of GAS, Stat5 was transfected into the cells. Stat5 transfected cells expressed higher levels of Stat5 than non-transfected cells and PRL, but not R1881, phosphorylated Stat5 (Figure 13A). With ectopic Stat5, PRL and R1881 alone failed to stimulate the 2.0-kbp CPD promoter, but when administered together they stimulated the activity of this promoter by more than 3-fold (Figure 13B). Similarly, ectopic Stat5 enhanced the effects of the suboptimal doses of PRL and R1881 on the 2.0-kbp CPD promoter activity (Figure 13B).

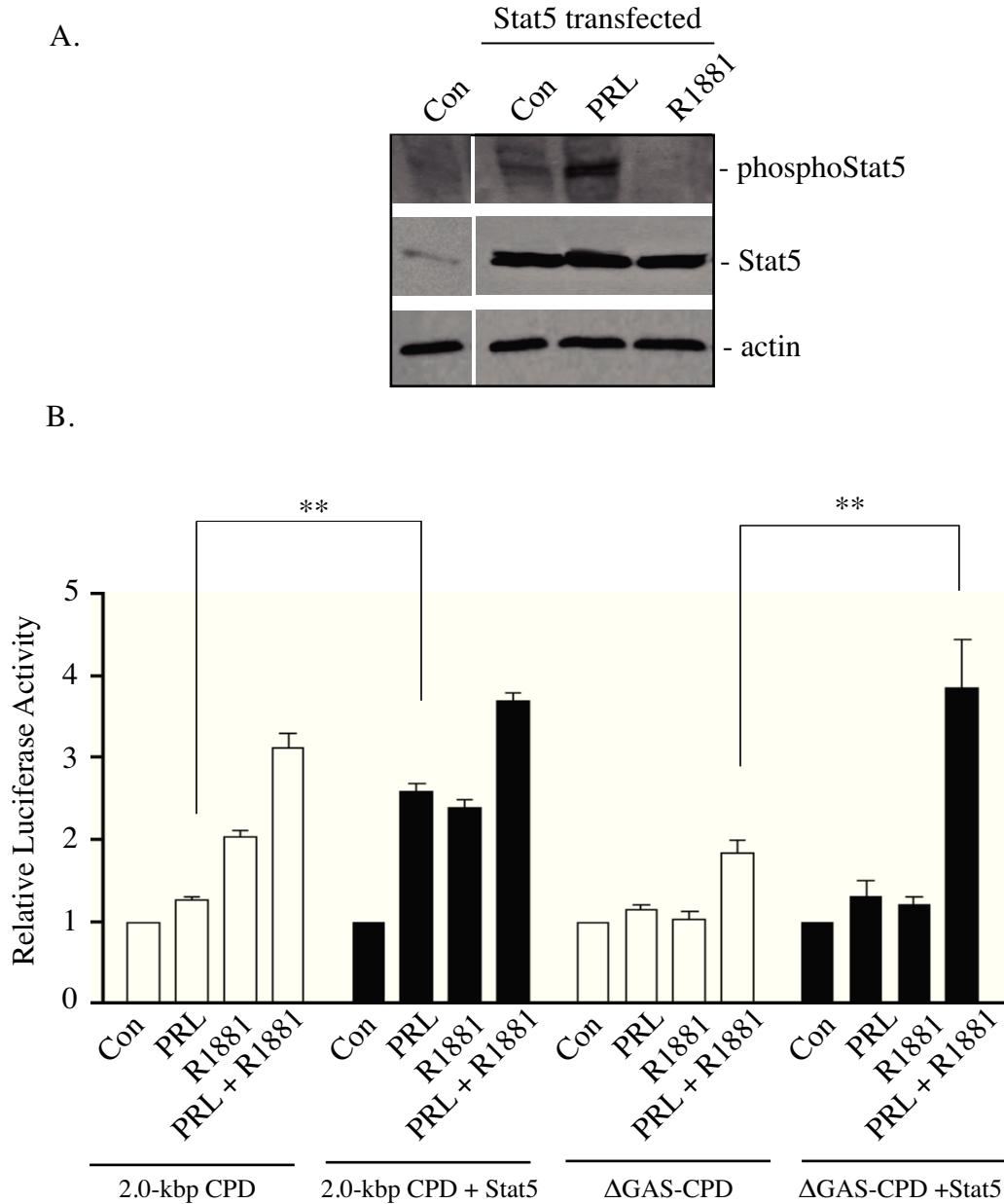


Figure 13. Sub-optimal doses of PRL and R1881 stimulate Δ GAS-CPD promoter activity. A, MCF-7 cells were transfected with Stat5 or left untransfected, growth arrested for 48 h, and then treated with either PRL or R1881 for 15 min. Control cells were left untreated. Cell lysates were used for western analyses to check Stat5 phosphorylation. B, MCF-7 cells were transfected with either the 2.0-kbp CPD or Δ GAS-CPD promoter construct, phRL-TK, and Stat5 construct as shown. After 5 h of transfection, the cells were growth arrested for 48 h, and then treated with either PRL (0.1 ng/ml) or R1881 (0.1 nM) alone or in combination (PRL + R1881) for 6 h. Cell lysates were used to measure luciferase reporter activity. Relative luciferase activity is presented as fold induction above baseline control which was arbitrarily set at 1. Three independent experiments were conducted in duplicates. Mean \pm SEM, n=3-4. **p<0.001.

These findings suggest that PRL-activated Stat5 might co-operate with the liganded androgen receptor to activate CPD gene transcription.

3.10 Stat5 gene knockdown abrogated the cooperative effect of PRL and R1881 on Δ GAS-CPD promoter activity

Next, the selective knockdown of the Stat5 gene in MCF-7 cells was performed using siRNA-targeting. Stat5 knockdown of the CPD gene was confirmed by RT-PCR and Western analysis. Stat5A mRNA levels was abolished for upto 72 h following siRNA transfection, but reappeared at 96 h. The decreased Stat5 protein levels at 72 h was also confirmed by Western analysis (Figure 14A).

The knockdown of Stat5, but not the non-targeting control (siNT), significantly reduced the cooperative effect of PRL and R1881 (Figure 14B) on the activity of the Δ GAS-CPD construct. The induction caused by ectopic Stat5 was effectively reduced by Stat5 knockdown, confirming the role of Stat5 in the cooperative stimulation of CPD gene transcription.

3.11 GAS and ARE-1 motif of CPD promoter are functional

PRL and R1881 stimulated the activities of CPD promoter constructs that contained both GAS and ARE-1. However, neither hormone stimulated constructs that contain a mutated GAS, whether by substitution or deletion. CHIP assays were next performed to determine whether the PRL-activated Stat5 and the liganded androgen receptor bound to the GAS and ARE-1, respectively. This procedure permits fine

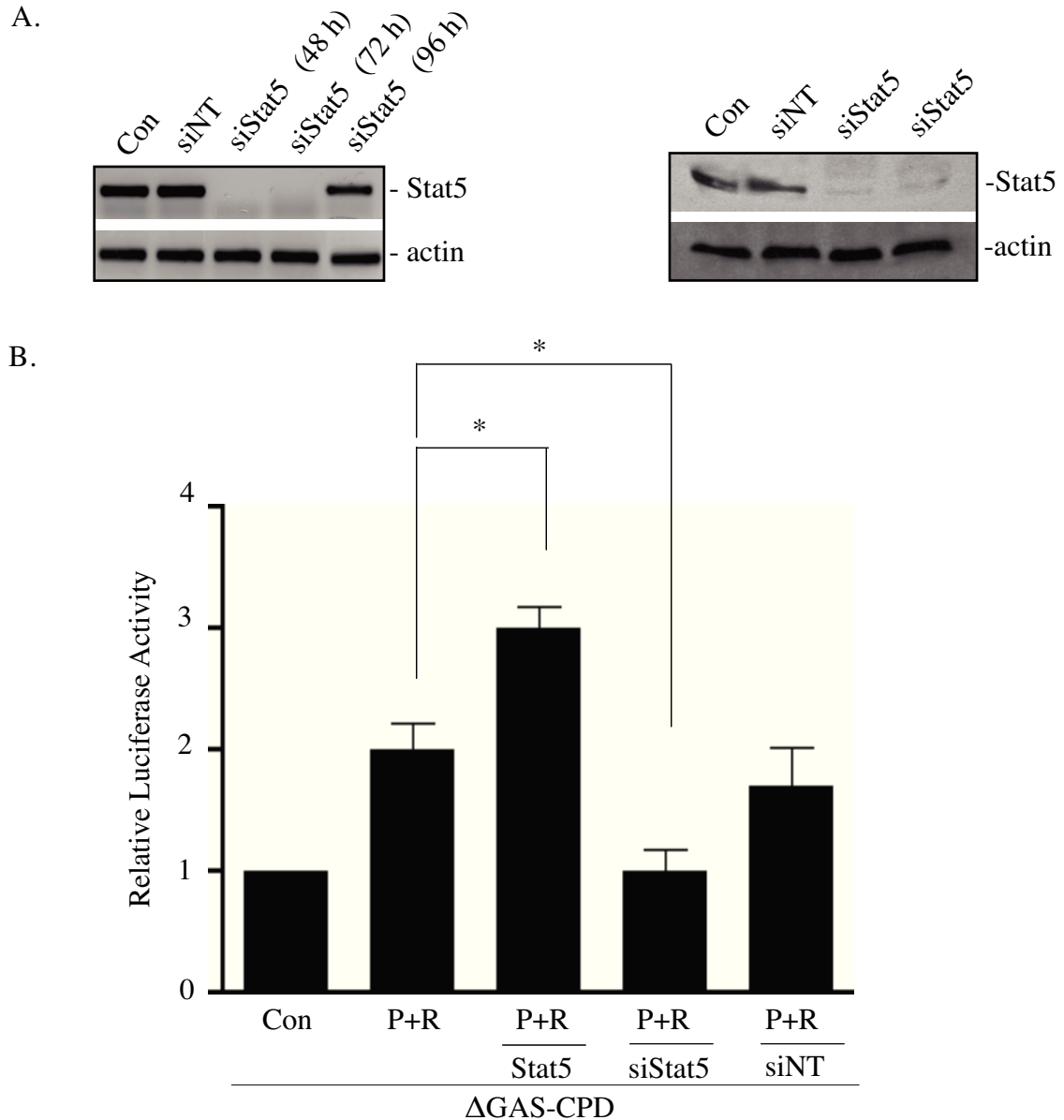


Figure 14. Stat5 siRNA inhibits PRL+R1881 stimulation of Δ GAS-CPD promoter activity. MCF-7 cells were transfected with 10 nM siNT and siStat5 as described in Materials and Methods. Control cells were not transfected. Cells were harvested at various timepoints for RT-PCR and western analyses. A, RT-PCR (right panel) and western analysis (left panel) showed the effective knockdown of Stat5 gene expression in siStat5-transfected cells. B, The Δ GAS-CPD promoter and renilla luciferase (phRL-TK) were co-transfected with Stat5, siStat5, or siNT as shown in the figure. After 5 h of transfection, the cells were growth arrested for 48 h, and then treated with both prolactin (P) (0.1 ng/ml) and R1881 (R) (0.1 nM) for 6 h. Controls were left untreated. Cell lysates were used to measure luciferase reporter activity. Relative luciferase activity is presented as fold induction above baseline control which was arbitrarily set at 1. Three independent experiments were conducted in duplicates. Mean \pm SEM, n=3. * P<0.05.

mapping of DNA sequences involved in the interactions with transcription factors while maintaining the integrity of their protein-DNA composition.

MCF-7 cells were starved in 1% CSSM for 48 h before treatment with PRL (10 ng/ml) or R1881 (10 nM). ChIP-ready chromatin was then prepared as described in Materials and Methods. Chromatin was then digested with micrococcal nuclease (2 U/ml) at increasing times and the digested DNA was collected (Figure 15B). These DNA fragments (100-300 bp) were then used in ChIP assays to determine the binding sites for the PRL-activated Stat5 and the liganded androgen receptor for the activation of CPD gene transcription.

Samples were prepared for ChIP assays. Briefly, MCF-7 cells were treated with PRL for 15 min and R1881 for 6 h. The cells were trypsinized for the crosslinking reaction. Nuclei were prepared for digestion with MNase. Five percent of total chromatin (5% input) after MNase digestion was used as a positive control to demonstrate that chromatin prepared by MNase digestion was amenable to ChIP analysis.

Immunoprecipitations were performed with anti-Stat5, anti-AR, anti-RNAPol II and IgG antibodies. Immunoprecipitations with RNA Pol II and IgG antibodies were used as a positive and a negative control, respectively. GAPDH primers were used to amplify the DNA immunoprecipitated by anti-RNA Pol II and IgG antibodies. The GAS and ARE-1 specific primers were used to amplify the DNA immunoprecipitated by anti-Stat5 and AR antibodies, respectively. DNA immunoprecipitated by anti-Stat5 and anti-AR antibodies was successfully amplified by GAS-ARE-1 primer demonstrating that PRL-activated Stat5 and liganded AR binds to GAS and ARE-1 region of CPD gene promoter. The GAPDH primer failed to amplify the DNA immunoprecipitated by Stat5 and AR

antibodies (Figure 15C). These results demonstrate that PRL-activated Stat5 and the liganded AR are bound to GAS and ARE-1 of the CPD gene promoter to stimulate CPD gene transcription.

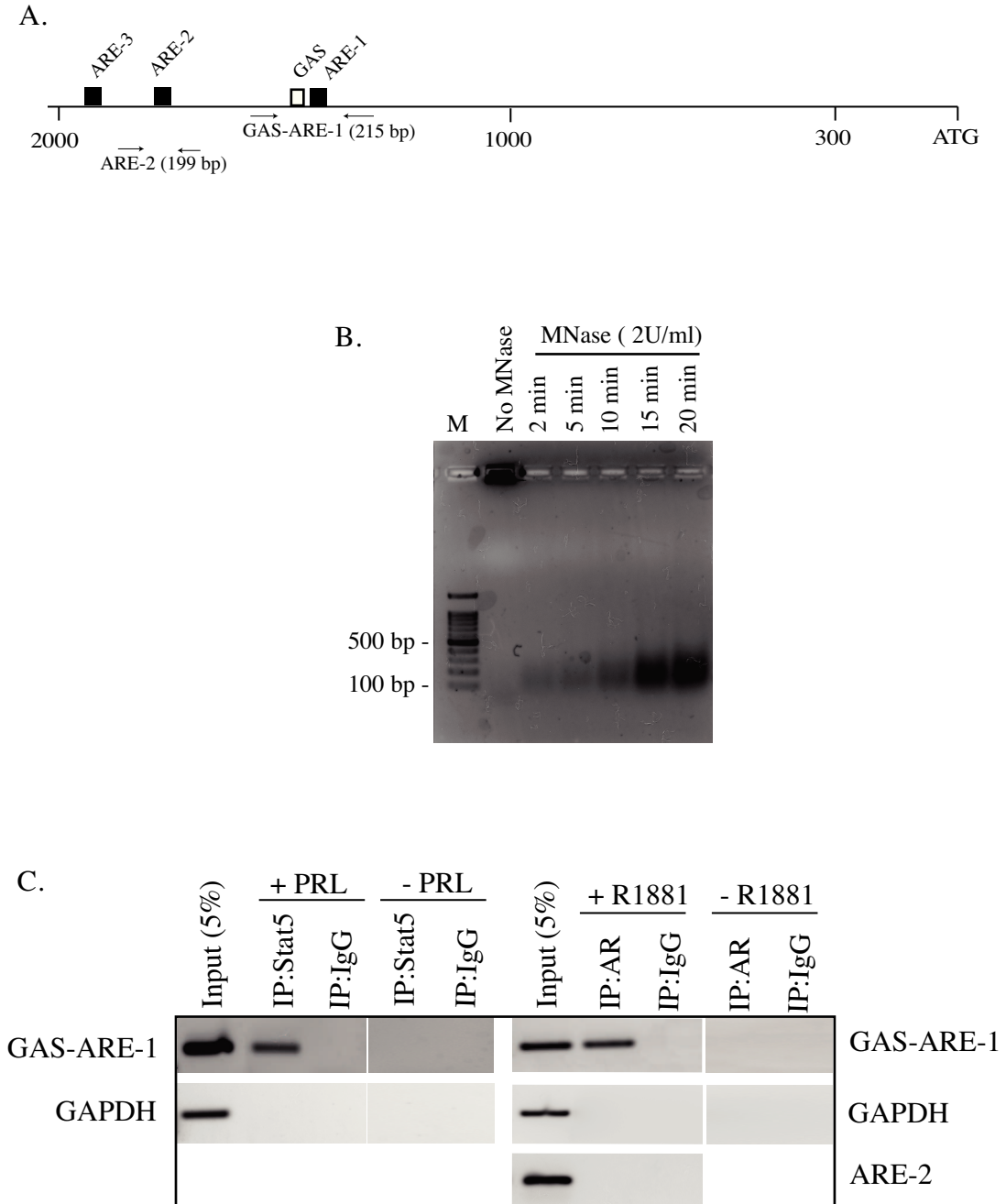


Figure 15. Chromatin immunoprecipitation analysis of the CPD promoters .

A) Depicted is the CPD gene promoter and ChIP assay primers. B) Optimization of MNase digestion parameters for digesting genomic DNA of MCF-7 cells. MNase (2 U/ml) was used to digest the DNA cross-linked with proteins. C) Quiescent MCF-7 cells treated with or without PRL (10 ng/ml) and R1881 (10 nM) were used for ChIP analyses. Immunoprecipitations were performed with anti-Stat5, anti-AR or IgG. Amplification of immunoprecipitated DNA was performed with primer sets as indicated. Representative of two independent experiments.

CHAPTER 4. DISCUSSION

4.1 Hormonal regulation of the CPD gene expression in breast cancer cells

Several studies have shown that CPD, by releasing C-terminal arginine from its substrates, contributes to NO production in RAW 264.7 macrophage, rat lung and microvascular endothelial cells (Hadkar, *et al.*, 2004; Hadkar and Skidgel, 2001). CPD mRNA levels were upregulated by lipopolysaccharide and IFN- γ (Hadkar, *et al.*, 2004; Hadkar and Skidgel, 2001), and by TGF- β in RAW-264.7 macrophages (Hoff, *et al.*, 2007). Our laboratory was the first to demonstrate that CPD is regulated by hormones in cancer cells. We have previously reported that E2 and PRL upregulate CPD mRNA and/or protein levels in human MCF-7 breast cancer and human HepG2 hepatoma cells (Abdelmagid and Too, 2008; Too, *et al.*, 2001). The increase in CPD levels in MCF-7 cells increases nitric oxide production, which in turn promotes cell viability and inhibits apoptosis (Abdelmagid and Too, 2008). Furthermore, we have reported that testosterone and PRL also upregulates CPD mRNA and protein levels, leading to the increased nitric oxide production for the survival of human prostate cancer cells (Thomas, *et al.*, 2012). In addition to E2 and progesterone, PRL and androgens are known to play critical roles in mammary tumorigenesis (see Introduction), each acting through its specific receptor to bring about specific cellular responses. The convergence of the PRLR, ER and AR signalling pathways on CPD gene activation is useful for the characterization of hormonal interactions and crosstalk, leading to cell survival. This study focuses on the molecular mechanisms regulating CPD gene transcription and expression in breast cancer cells.

4.1.1 PRL, E2 and R1881 upregulate CPD gene expression in breast cancer cells

The present study for the first time demonstrates that R1881, in addition to E2 and PRL, upregulates CPD mRNA levels in a dose-dependent manner in MCF-7 cells. Maximum upregulation was seen at a dose of 10 μ M for the steroid hormones and 10 ng/ml of PRL (Figure 5). E2, PRL and R1881 also increase CPD protein levels in a time-dependent manner in both MCF-7 and T47D breast cancer cell lines (Figure 6). The action of these hormone are mediated by their specific receptors and transcription factors, suggesting that the presence of their respective response elements in the CPD promoter.

PRL exerts its effects via the PRLR, which activates Jak2 kinase, leading to the recruitment and activation of Stat proteins. Stat5a plays a more prominent role than Stat5b in PRLR signal transduction in the mammary gland (Hennighausen and Robinson, 2001). In mammary cells, the PRL-activated Stat5 proteins bind to the GAS sequence in the promoter region of target genes, some of which are involved in cell proliferation, differentiation, or motility (Clevenger, *et al.*, 2003). Analysis of the CPD promoter region reveals a consensus GAS, the Stat-binding motif, thereby implicating the involvement of the Jak-Stat pathway in CPD gene expression.

There is no consensus ARE in the CPD promoter for the binding of the liganded AR. However, some reported active AREs contain non-consensus bases to which the liganded AR can bind to initiate the transcription of androgen-regulated genes. For example, each of the three active AREs in the human protease-activated receptor-1 (hPar1) gene promoter contains eight non-consensus bases (Salah, *et al.*, 2005). Probasin (Qi, *et al.*, 2008) and prostate-specific antigen (PSA) (Riegman, *et al.*, 1991) gene

promoters also have non-consensus but active AREs (Figure 10). The CPD gene promoter has 3 putative non-consensus AREs within the 2.0-kbp region upstream from the start site (Figure 10). The binding of the liganded AR to one or more of these putative AREs could be a mechanism by which androgens activate the CPD gene in breast cancer cells.

The CPD gene promoter does not contain any consensus ERE. However, the nongenomic actions of estrogen may indirectly turn on gene expression, through the activation of cytoplasmic kinase pathways (e.g. MAPK), and/or through protein-protein interactions of the ER with other transcription factors (e.g., Sp1) in the nucleus. For example, the ER physically interacts with transcription factor Sp1 to stimulate the estrogen-dependent vitellogenin A1 gene promoter (Batistuzzo de Medeiros, *et al.*, 1997). Furthermore, the relative positions of the ERE and Sp1-binding sites, with respect to the transcription initiation site, could determine whether the ER and Sp1 synergizes to regulate transcription initiation (Batistuzzo de Medeiros, *et al.*, 1997). Similarly, crosstalk between the ER and transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) has been reported in breast cancer cells, to enhance the expression of cell survival genes such as *PHLDA1* and *BIRC3* (Frasor, *et al.*, 2009). Since, the CPD gene promoter contains binding sites for Sp1 and NFκB, the liganded ER could potentially regulate CPD gene transcription through these transcription factors.

4.1.2 PRL and R1881, but not E2, activate CPD gene transcription

Hormonal regulation of target gene expression can be at the transcriptional and/or translational level. PRL and R1881 upregulation of CPD mRNA levels were suppressed

by a transcriptional inhibitor, actinomycin D, suggesting that these two hormones directly activate CPD gene transcription. However, the increase in CPD mRNA levels following E2 treatment was not affected by actinomycin D (Figure. 7), leading to the speculation that E2 could have other effects, such as regulation of CPD mRNA stability. In fact, many hormones are known to prolong the half-lives of their target mRNAs. For example, the stabilities of the mRNAs encoding thyrotropin-releasing hormone receptor, casein, AR, and the progesterone receptor have previously been shown to be stabilized by estrogen, PRL, DHT, and follicle stimulating hormone, respectively (Brock and Shapiro, 1983; Guyette, *et al.*, 1979; Iwai, *et al.*, 1991; Yeap, *et al.*, 1999). The best-known example is the estrogen protection of *Xenopus* liver vitellogenin mRNA against cytoplasmic degradation. Estrogen increases both the transcriptional activity of the vitellogenin gene and the stability of the vitellogenin mRNA. The half-life of vitellogenin mRNA increases dramatically from 16 h to 500 h after the administration of estrogen (Brock and Shapiro, 1983).

4.1.3 E2 promotes CPD mRNA stability in MCF-7 cells

The stability of mRNAs could be affected by response to hormonal, environmental, and developmental factors (Wilson and Brewer, 1999). These factors could increase the stability of target mRNAs and change the cytoplasmic mRNA concentration without any change in transcriptional activity. The stability of mRNAs is attributable to the presence or absence of specific sequence elements, which include the well-characterized adenosine (A) and uridine (U)-rich elements located in the 3'-untranslated regions (UTRs) of mammalian mRNAs. In addition, the 5'- and 3'-terminal

nuclear modifications of eukaryotic mRNAs, the cap (7mGpppN) and poly (A) tail, respectively, play critical roles in mRNA translation and stability.

The upregulation of the CPD mRNA by E2 in MCF-7 cells appears to be due to an increase in mRNA stability (Figure 9). The cis-acting element and RNA binding proteins that are potentially involved in E2 regulation of CPD mRNA stability are yet unknown. Furthermore, CPD mRNA levels were unaffected by the administration of protein synthesis inhibitor cycloheximide, suggesting that transcriptional upregulation of the CPD gene by E2, PRL and R1881 does not require *de novo* protein synthesis (Figure 8).

4.2 PRL and R1881, not E2, stimulate CPD promoter activity

The studies above have established the roles of E2, PRL and R1881 in the activation of CPD gene expression in breast cancer cells. The CPD gene promoter contains potential binding sites for PRL-activated Stat5 and the liganded AR. Therefore, I proceeded to characterize the hormonal regulation of CPD gene promoter activity, and to identify the active transcription factor binding sites in the promoter.

Luciferase reporter constructs containing the 0.7-, 2.0-, or 7.0-kbp CPD gene promoter region were all transcriptionally active when transfected into HepG2 and Chinese Hamster Ovary cells (Timblin, *et al.*, 2002). My study showed that these constructs were also active when transfected into MCF-7 cells (Figure 11A). The 2.0-kbp CPD gene promoter was activated by PRL and R1881, but not E2 (Figure 11B), implicating that the identified GAS and at least one of the putative AREs were active. The failure of E2 to activate the 2.0-kbp CPD-promoter could be due to the lack of any

ERE in this region. This finding is in agreement with the actinomycin D experiments showing that this transcriptional inhibitor had no effect on E2 action (Figure 7).

None of the hormones had any effect on the activity of the 0.7- and 7.0-kbp CPD promoter constructs (Figure 11B). Their inability to activate the 0.7-kbp CPD promoter was probably due to the absence of GAS, ARE and ERE motifs. However, the 7.0-kbp promoter which contains GAS and the several potential AREs also did not respond to any of these hormones. Proper folding of the endogenous DNA is essential to facilitate binding of co-regulators to gene promoters. Proper looping of DNA would allow co-regulators to interact with transcription factors binding response elements in gene promoters. However, there is a possibility that when a very large promoter sequence (e.g. 7.0-kbp) is cloned into a vector, it loses its structural integrity and its ability for endogenous interactions with coregulators. This could explain the lack of activation of the 7.0-kbp-promoter construct by PRL and R1881. Also, the transfection procedure for the CPD gene promoter was optimised for the 2.0-kbp construct. Therefore, equal amounts of the 7.0-kbp and 2.0-kbp promoter DNA were used, less copies of the 7.0-kbp promoter would be transfected as compared to the 2.0-kbp promoter. The actual amount of 7.0-kbp promoter DNA transfected into the cells might not have been enough to observe any promoter activity.

4.3 GAS mutation suppresses PRL and R1881 activation of the 2.0-kbp CPD gene promoter

If the putative AREs were active, their mutation would abrogate R1881 activation of the CPD promoter. Likewise, mutation of the GAS sequence in the CPD gene

promoter was expected to abolish its activation by PRL. PRL, but not R1881, phosphorylate and activate Stat5 in MCF-7 cells. The activated/phosphorylated Stat5 binds to the GAS motif to activate gene transcription. The liganded AR does not bind to GAS and, therefore, R1881 activation of the promoter should not be affected by a GAS mutation.

Figure 12B showed that PRL and R1881 continued to stimulate the 2.0-kbp promoter construct even with the deletion of ARE-2 and ARE-3 (Δ ARE2,3-CPD). This observation indicated that R1881 stimulation of CPD promoter activity was independent of ARE 2 and ARE 3, but dependent on ARE-1. The stimulatory effect of PRL was likely due to the presence of the identified GAS. When ARE-2, ARE-3 and GAS were all mutated, neither PRL nor R1881 activated the promoter. Similarly, neither PRL nor R1881 activated the promoter (Δ GAS-CPD) containing a GAS mutation but with an intact ARE-1, Δ GAS-CPD (Figure 12B). The lack of a response with R1881 treatment was unexpected, suggesting that an intact GAS was required for the liganded AR to bind on ARE-1.

4.4 Cooperative action of PRL and R1881 on CPD promoter activity

There is considerable evidence of cooperativity between Stat proteins and other transcription factors that are bound to neighboring sites in the same promoter region. For e.g. Intercellular Adhesion Molecule 1 (ICAM1) gene promoter contains GAS site in close proximity of Sp1 site, and binding of both Stat and Sp1 is required for full activation of the gene. Co-immunoprecipitation and ChIP assay showed they are in complex when activating ICAM1 gene transcription (Bowman, *et al.*, 2000; Chatterjee-

Kishore, *et al.*, 2000). In addition to direct binding to the GAS motif, Stat proteins can bind to non-GAS site to cooperate with other non-Stat transcription factor(s) (Shuai, 2000). Alternatively, Stat proteins can also cooperate with other transcription factors that enhance Stat5-GAS binding to activate target gene transcription (Shuai, 2000). Studies by Carsol *et. al.*, showed that the PRL-activated Stat5 and the DHT-activated AR stimulate target gene transcription in a synergistic manner in breast cancer cells. The trans-activation domains of Stat5 and AR act synergistically to activate Stat5 and AR signalling pathways (Carsol, *et al.*, 2002). Active Stat5 has also been shown to physically interact with the liganded AR, and each increases the nuclear localization and transcriptional activity of the other (Tan, *et al.*, 2008). The finding that R1881 alone does not activate the Δ GAS-CPD gene promoter, with an intact ARE-1, prompted me to test the hypothesis that the AR and PRL-Stat5 signalling pathways work cooperatively in activating CPD gene transcription.

4.4.1 Suboptimal doses of PRL and R1881 together activate Δ GAS-CPD promoter activity.

To investigate potential interactions between the PRL-Stat5 and AR signalling pathways, suboptimal doses of PRL and R1881 were tested together on Δ GAS-CPD promoter activity in MCF-7 cells. PRL and R1881 together, but not alone, stimulated the activities of the 2.0-kbp CPD and Δ GAS-CPD promoter constructs (Figure 13B), implicating the cooperation between PRL-Stat5 and the liganded AR. Furthermore, when Stat5 was ectopically overexpressed in the transfected cells, PRL and R1881 in combination activated both the 2.0-kbp CPD and Δ GAS-CPD promoters to a higher

degree, demonstrating the cooperation between Stat5 and the liganded AR. Our laboratory has also shown that PRL and R1881 act synergistically to promote NO production in breast cancer cells, which was abrogated by the combination of PRLR and AR antagonists (Thomas and Too, unpublished).

4.4.2 siStat5 inhibits the cooperative stimulation of PRL and androgen on Δ GAS-CPD promoter activity

To confirm the involvement of Stat5 in promoting the cooperative action of PRL and androgen on Δ GAS-CPD promoter activity, the endogenous Stat5 was knocked down using siRNA. Western blot and qPCR analyses confirmed that the siRNA treatment resulted in the efficient knockdown of Stat5 gene expression (Figure 14A). The knockdown of Stat5 significantly reduced the synergistic effects of PRL and R1881 (Figure 14B), confirming the essential role of Stat5 in the cooperative stimulation of the CPD gene transcription by PRL and R1881.

4.4.3 The CPD gene promoter GAS and ARE1 motifs are functional

To further confirm the results obtained by luciferase reporter assays, ChIP analyses were conducted to determine whether the PRL-activated Stat5 and the liganded AR bind to GAS and ARE-1, respectively. The primers flanking the GAS and ARE-1 motifs of the CPD gene promoter successfully amplified the DNA fragments immunoprecipitated by anti-Stat5 and AR antibodies, respectively (Figure 15C), confirming the validity of the GAS and ARE-1 binding sites. The primers flanking ARE-2 region did not amplify the DNA immunoprecipitated by anti-AR antibodies (Figure

15C), supporting the previous finding that ARE-2 was not active, that is, it was not a binding site for the liganded AR.

4.5. Conclusion

This study demonstrated that E2, PRL, and R1881 upregulated CPD protein and mRNA levels in a time- and dose-dependent manner. The increase in CPD mRNA levels was found to be transcriptionally regulated by PRL and R1881, since actinomycin D inhibited the stimulatory effect of these hormones. In contrast, E2 upregulation of CPD mRNA levels was not inhibited by actinomycin D, but was due to an increase in CPD mRNA stability. The activity of the 2.0-kbp CPD promoter was increased by the administration of PRL and R1881, implicating the involvement of the GAS motif and at least one of the AREs found in the promoter region. The failure of E2 to activate the 2.0-kbp CPD promoter was consistent with the absence of an ERE motif, as well as the earlier finding that the E2-mediated increase in CPD mRNA levels was not inhibited by actinomycin D. The deletion of ARE-2 and ARE-3 did not affect the activation of the CPD promoter by PRL and R1881, suggesting that GAS and ARE-1, but not ARE-2 nor ARE-3, were functional. The PRL and R1881 stimulation of the Δ GAS-CPD promoter, with an intact ARE-1, were abolished upon deletion of the GAS motif, but the activity was restored when PRL and androgen were administered together, implicating cooperative action between the PRL-activated transcription factor Stat5 and the liganded AR. The cooperative effect of PRL and androgen was further enhanced in the presence of ectopic Stat5, providing additional evidence for the involvement of the PRL-Stat5 pathway in CPD gene transcription. ChIP analysis showed that the PRL-activated Stat5

and the liganded AR bound to the GAS and ARE-1 motifs in the CPD promoter, respectively, thus confirming PRL and R1881 stimulation of CPD gene transcription through Stat5 and AR.

In summary, the findings discussed above provide insight into the mechanisms by which E2, PRL and R1881 promote CPD gene transcription and expression. This study also demonstrates the convergence of PRL- and androgen-activated pathways in the transcriptional activation of the CPD gene (Figure 16). Our laboratory has previously shown that hormonal upregulation of CPD increases NO production, which in turn leads to the survival of MCF-7 breast cancer cells (Abdelmagid and Too, 2008). As explained in the Introduction, E2, PRL and R1881, acting through their specific receptors can also regulate other genes to bring about breast tumourigenesis. Therefore, instead of targeting a myriad of hormone-regulated targets, targeting the PRLR and the AR, in addition to the ER, would be an important strategy for the treatment of breast tumour that express these receptors.

The convergence of PRLR and AR signalling pathways in the cooperative stimulation of CPD gene transcription in mammalian cells in culture could be further explored in mouse models. For example, a xenograft model of MDA-MB-453 (triple negative for ER, PR and HER2, but expressing AR and PRLR) could be established to determine the effect of targeting the PRLR and AR with receptor antagonists Δ G129hR-hPRL and bicalutamide, respectively. The MDA-MB-453 cells would be grown as solid mammary tumours in NOD/SCID mice. The mice bearing tumours would be treated with daily administration of vehicle, or with AR and PRLR antagonists, bicalutamide and Δ G129hR-hPRL, respectively, either alone or in combination. Tumour volume would be

determined and compared between vehicle- and drug-treated mice. The effect of receptor antagonists could be assessed on several parameters, such as tumor growth and volume, and immunostained for proliferative and angiogenic markers. Recently, a new PRLR antagonist LFA102 (Diamiano, *et al.*, 2012) has been put on phase I preclinical trial by Novartis, for patients with PRLR-positive metastatic breast cancer (<http://clinicaltrials.gov/show/NCT01338831>). In addition, a multicenter phase II trial (NCT00468715) of the AR antagonist, bicalutamide, for patients with AR-positive ER-/PR- metastatic breast cancer, was found to be well tolerated, and prolonged life by > 6 months in that treatment group (Thanopoulou, *et al.*, 2013).

In summary, this study suggests that targeting the PRL and androgen receptor signalling pathways would be an important consideration and an additional strategy for the treatment and/or management of breast cancer. Such therapies that target the PRLR and AR could be administered to patients that have AR or PRLR positive breast cancers.

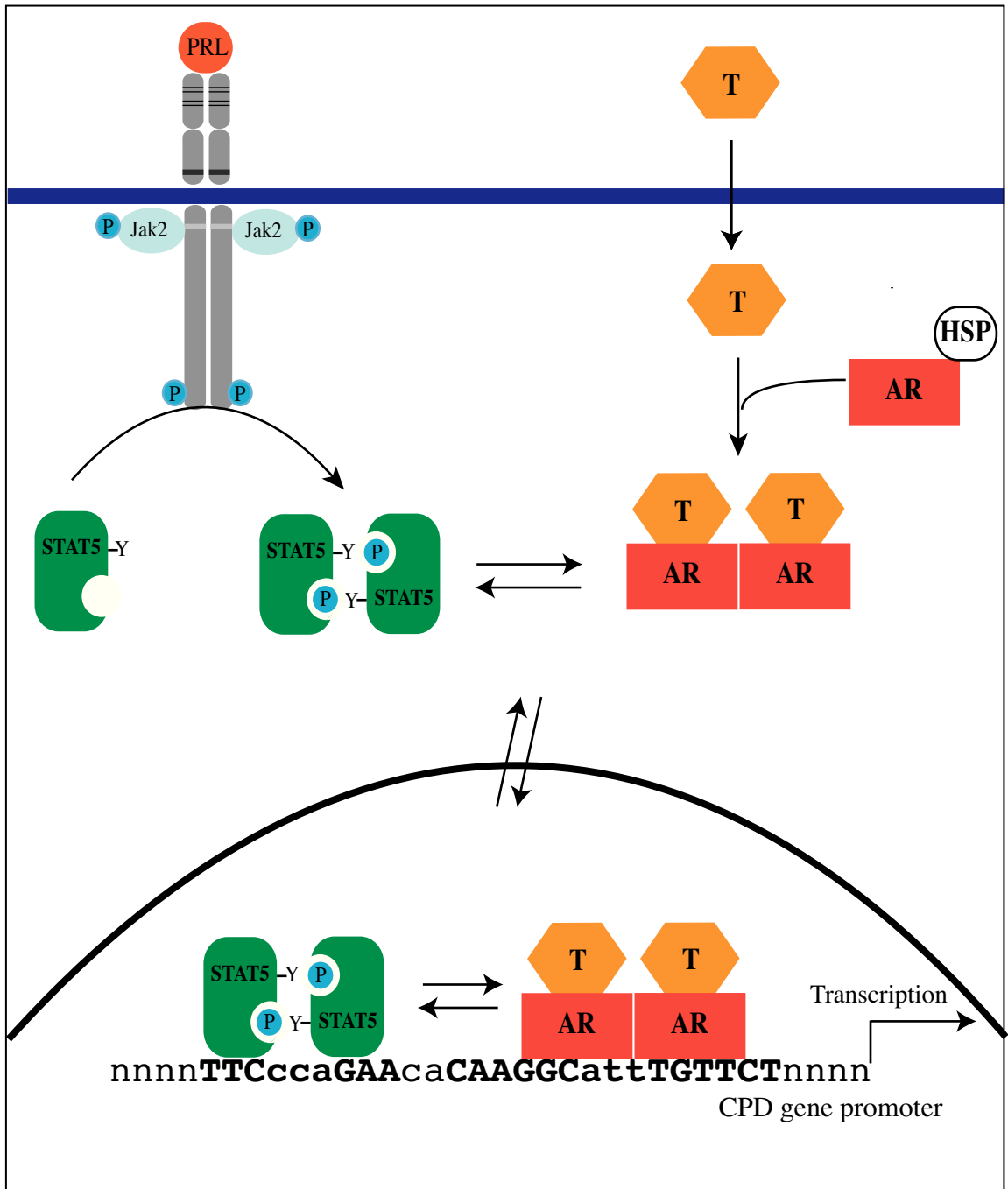


Figure 16. Functional cooperation of Stat5 with liganded AR to activate CPD gene transcription. In the presence of PRL and androgen, the PRL-activated Stat5 dimer cooperates with the liganded androgen receptor to activate CPD gene transcription.

4.6 Future Studies

The present study has provided insight into the regulation of CPD gene transcription and expression by PRL, E2 and R1881. This study confirmed the validity of the GAS and ARE-1 motifs in the CPD gene promoter, and also demonstrated the cooperative effect of PRL and R1881 on CPD promoter activity. The present findings have opened new avenues for future cancer research. The potential physical interaction between the PRL-activated Stat5 and the liganded AR for the activation of CPD gene expression was not determined in this study. Stat5 and AR have been reported to interact physically in prostate cancer cells (Tan, *et al.*, 2008) but have yet to be shown in breast cancer cells. Another future study would be to determine the effect of Stat5 on the nuclear localization of the liganded AR, and vice versa, in breast cancer cells.

E2 upregulation of CPD gene expression was determined to occur non-transcriptionally. Thus, it would be of interest to determine whether any other transcription factors (e.g., Sp1) or signalling pathways (e.g., MAPK) are involved in E2-regulated CPD gene expression. Another future study could be to identify cis- or trans-acting elements and RNA-binding proteins that is/are involved in E2-mediated CPD mRNA stability. For example, DNA elements of untranslated regions (UTR) like 5' cap region, 3' poly-A tail and 3' adenine and uridine (AU)-rich elements are known to be involved in mRNA stability and degradation. Similarly, RNA binding proteins like AU-rich element binding factor and iron regulatory protein has been shown to be involved in mRNA stabilization (Guhaniyogi and Brewer, 2001).

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