Mechanism of Cyclin D1 Regulation by Progestins in Breast Cancer

by

Shweta Krishnan

Department of Pharmacology and Cancer Biology
Duke University

Date:_____

Approved:

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Donald McDonnell, Supervisor

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Gerard Blobe

_____
Jeffrey Rathmell

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Ann Marie Pendergast

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Jeffrey Marks

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
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2014

ABSTRACT

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Abstract

The majority of breast tumors express the estrogen receptor (ER), and more than half of these cancers also express the progesterone receptor (PR). While the actions of ER on breast cancer pathogenesis are well understood, those of PR are still unclear. The Women's Health Initiative trial in 2002 brought into focus the alarming result that women receiving both estrogen and progestins as hormone replacement therapy are at greater risk for breast cancer than women receiving estrogen alone. Thus, there is considerable interest in defining the mechanisms that underlie the pharmacological actions of progestins in the normal and malignant breast.

Progestins facilitate cell cycle progression through multiple mechanisms, one of which is the induction of phosphorylation of the tumor suppressor retinoblastoma (Rb) protein. Stimulation by growth factors induces the transcription of Cyclin D1 which in turn activates the cyclin dependent kinases (CDKs). The Cyclin D1- Cdk4/6 complex phosphorylates the Rb protein, leading to the release of E2F1, which then binds and activates other target genes, leading to G1-S transition of the cell cycle. Given the reported action of PR to activate MAPK signaling, we initially thought that the progestin-induced Rb phosphorylation was mediated by this pathway. However, we turned to an alternate hypothesis based on our data using MEK inhibitors demonstrating that this was not the case.

Given the primacy of Cyclin D1 in cell cycle control, we then turned our attention to defining the mechanism by which Cyclin D1 expression is regulated by PR.

Interestingly, it was determined that progestin mediated up- regulation of Cyclin D1 is rapid, peaking at 6hrs post hormone addition followed by a decrease in expression reaching a nadir at 18hrs. Unexpectedly, we found that contrary to what has been published before, the induction of Cyclin D1 mRNA expression was a primary transcriptional event and we have demonstrated the specific interaction of PR with PREs (progesterone response elements) located on this gene. We have further determined that the half-life of Cyclin D1 mRNA is decreased significantly by progestin addition explaining how the levels of this mRNA following the addition of hormone are quickly attenuated. Thus, when taken together, our data suggest that progestins exert both positive and negative effects on Cyclin D1 mRNA, the uncoupling of which is likely to impact the pathogenesis of breast cancer

The observation that PR reduces the Cyclin D1 mRNA stability led us to investigate the effects of PR on RNA binding proteins, especially those which are involved in RNA stability. We discovered that PR induces the expression of several RNA binding proteins. Although the work to determine the effects of these RNA binding proteins on CyclinD1 mRNA stability is still ongoing, we have discovered a role for one of the PR-induced RNA binding proteins tristetraprolin (TTP), in the suppression of the inflammation pathway in breast cancer. We found that while TTP was not required for the PR-mediated decrease in Cyclin D1 mRNA stability, overexpression of this tumor suppressive protein was able to inhibit IL-1β-mediated stimulation of inflammatory genes in our breast cancer model. Since it is established that the upregulation of the

inflammatory pathway is oncogenic, we are currently exploring the intersection of PR and TTP-mediated signaling on the inflammation transcriptome in breast cancer.

Thus, collectively these data provide us with a better picture of the poorly understood actions of PR on breast cancer proliferation and tumorigenesis. We believe that further investigation of the studies developed in this thesis will lead to novel and better-targeted approaches to the use of PR as a therapeutic target in the clinic.

Dedication

This thesis is dedicated to the perseverance of my parents, whose dream it was to give their daughters an education beyond the norm.

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Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
ActD	Actinomycin D
AF	Activation function
AP-1	Activator protein
AR	Androgen receptor
BUS	B-upstream segment
C	Carboxy (-terminus)
cAMP	Cyclic adenosine monophosphate
CBP/p300	CREB-binding protein
CDK (2 and 4)	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CoA	Coactivator
CS-FBS	Charcoal-stripped fetal bovine serum
CTE	Carboxy terminal extension
DBD	DNA binding domain
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle medium

DNA	Deoxyribonucleic acid
E2	17β-estradiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ER (α and β)	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FKBP51	FK506-binding immunophilin 51
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GO-term	Gene ontology term
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
h	Hinge region
HABITS	Hormonal replacement therapy after breast cancer: is it safe?
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMEC	Human mammary epithelial cell
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy

HSP	Heat shock protein
ID	Inhibitory domain
IgG	Immunoglobulin G
IRES	Internal ribosome entry site
JAK	Janus family of tyrosine kinases
kDa	Kilodalton
LBD	Ligand binding domain
LH	Luteinizing hormone
Luc	Luciferase
Lys	Lysine
MAPK	Mitogen activated protein kinase
MEKK1	MAPK kinase kinase 1
MEM	Minimum essential medium
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleic acid
N	Amino (-terminus)
NaPyr	Sodium pyruvate
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa B
NR	Nuclear receptor

P4	Pregn-4-ene-3,20-dione (or progesterone)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/streptomycin
PI3K	Phosphotidylinositol (PI) 3-kinase
PR	Progesterone receptor; h=human, c=chicken, x=*Xenopus*, m=membrane, g=genomic
PRE	Progesterone response element
PRM	Progesterone receptor modulator
PVDF	Polyvinylidene fluoride
PWM	Position weight matrix
qPCR	Quantitative real time polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
S100P	S100 calcium binding protein P
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH3	Src homology 3
siRNA	Small interfering ribonucleic acid
S-phase	Synthesis phase

SRC-3	Steroid receptor coactivator-3
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
SV40	Simian vacuolating virus 40
Thr	Threonine
WHI	Women's Health Initiative
β-gal	β-Galactosidase

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companionship, unconditional love, encouragement and positive energy every step of the way. Thank you for being my sanity and strength these past few months! I look forward to our journey together.

1 Background

1.1 Thesis research

The main goal of my studies is to investigate the mechanisms of progesterone receptor action in breast cancer. These studies will enable us to better understand PR biology and direct the development of therapeutics in the clinic to treat PR positive breast cancer.

1.2 Progesterone: biosynthesis

Progesterone is a naturally occurring steroid hormone that has an established role in the normal development and regulation of the female reproductive system. It functions by binding to the progesterone receptor (PR) and thereby enabling the receptor to bind DNA, recruit cofactors, and induce the transcription of target genes. Cholesterol derived from high or low-density lipoproteins is the precursor for steroidogenesis of progesterone. The free cholesterol is transported to the mitochondria in a rate-limiting step with the help of sterol carrier proteins (Figure 1.1). It has been demonstrated that steroidogenic acute regulatory protein (StAR) can bind cholesterol in the cytosol and transport it to the mitochondrial membrane where peripheral-type benzodiazepine receptors (PBR) move it to the inner mitochondtrial membrane. Further studies have shown that the transport of cholesterol can be regulated by the phosphorylation of StAR by kinases such as protein kinase A and protein kinase C. Cholesterol is then converted to pregnenolone by the cytochrome p450 cholesterol side chain cleavage complex. 3β-

hydroxysteroid dehydrogenase (3βHSD) then converts pregnenolone to progesterone in the smooth endoplasmic reticulum (Reviewed in (1)).

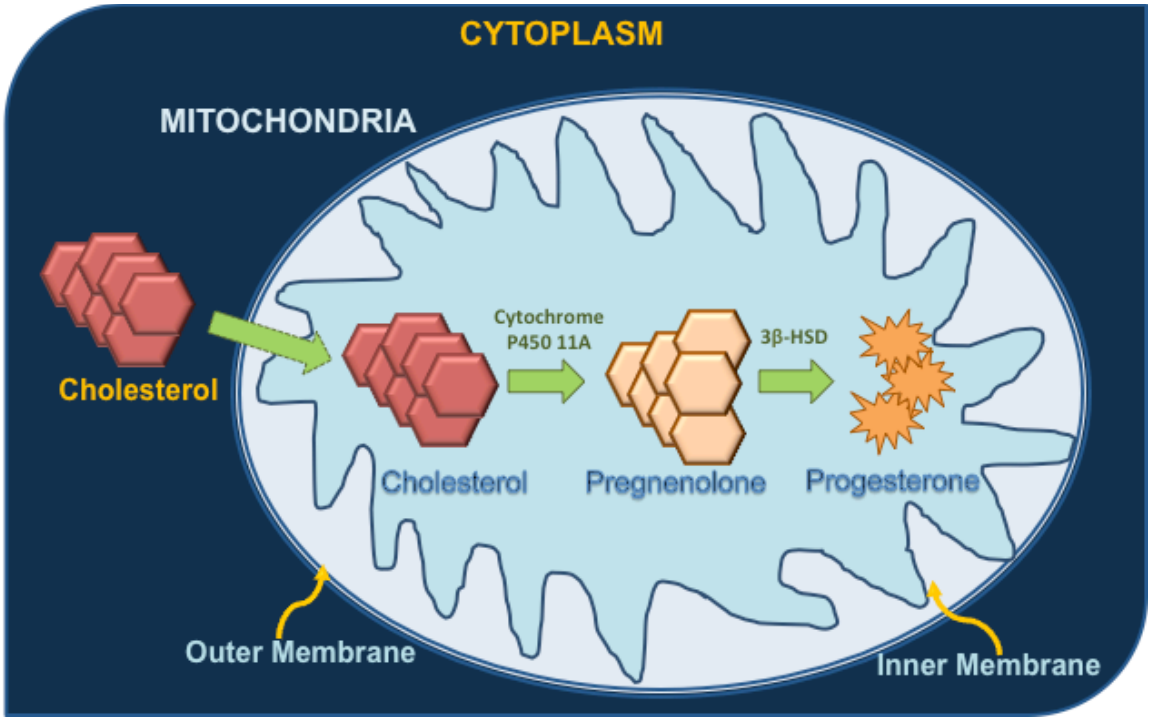


Figure 1.1: Progesterone biosynthesis

Figure depicting the transport of cholesterol from the cytosol to the mitochondrion and subsequent conversion to progesterone.

The primary body responsible for the production of progesterone during the female reproductive cycle and during pregnancy is the corpus luteum, which forms from residual follicular granulosal and thecal cells after ovulation. Luteinizing hormone (LH) - a glycoprotein hormone whose release is regulated by the pituitary gland, stimulates the secretion of progesterone in the small luteal cells of the mature corpus luteum. Luteinizing hormone binds to its cognate receptor, activating protein kinase (PK) A, ultimately increasing synthesis of StAR and the activity of cytochrome P450 11A and 3β-HSD, followed by increase of progesterone secretion (2-4).

Progesterone has also been shown to regulate it's own synthesis in several mammals (Reviewed in (5)). For instance, treatment of bovine luteal slices with progesterone stimulated 3β-HSD activity to levels comparable to luteinizing hormone; an effect that was inhibited by PR antagonists (6). Progesterone was able to upregulate the expression of StAR protein, cytochrome p450 and 3β-HSD in bovine luteal cells (7) which thus enabled it to participate in a positive feedback loop of its synthesis in luteal cells (6).

1.3 Physiological roles of progesterone

1.3.1 Role of progesterone in mammary gland development

The steroid hormone progesterone plays a central role in the female reproductive cycle, pregnancy establishment and maintenance. Progesterone is necessary to elicit the reproductive responses necessary for female fertility, as well as to elicit normal proliferative responses of the mammary gland (8). Using PR knockout (PRKO) mouse it

was demonstrated that progesterone is essential for pregnancy-associated mammary gland ductal side-branching and alveologenesis that are mediated by progesterone-regulated mammary epithelial proliferation. PR-dependent biology of the mammary gland has been proposed to rely on specific spatial organizational patterns of PR expression and deregulation of these molecular processes may contribute to abnormal mammary development as well as cancer (9).

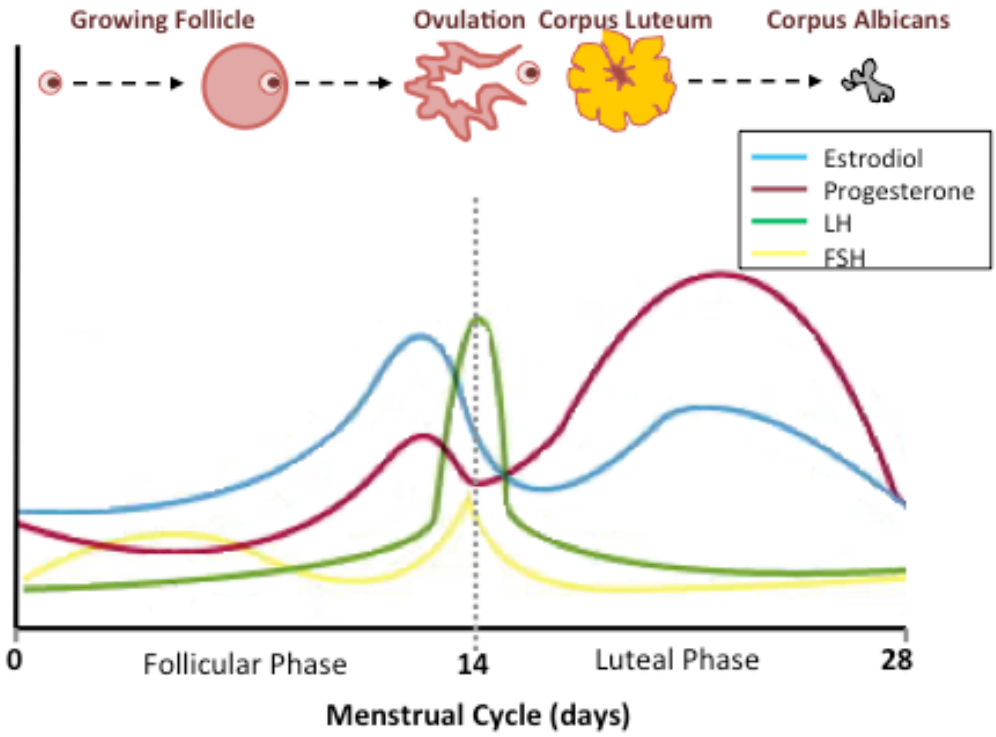


Figure 1.2: Female menstrual cycle

Graph depicting variations in hormone levels and development of a follicle over the 28-day menstrual cycle.

1.3.2 Progesterone and the menstrual cycle

The typical female menstrual cycle lasts for a duration of 28 days and consists of a large network of hormones that coordinate with each other to regulate the fate of a mature follicle (Figure 1.2). The cycle consists of two broad phases- follicular phase, characterized by the growth and development of a mature follicle, followed by the luteal or secretory phase that occurs after ovulation at the end of the first phase. The first day of the menstrual cycle is defined as the first day of menstruation when estrogen and progesterone secretions are withdrawn and follicle stimulating hormone (FSH) levels have increased as a result of loss of suppression by negative feedback. FSH then stimulates the growth and differentiation of a primordial follicle into a pre-ovulatory mature follicle, a process that can take in excess of 120 days, during which steroid production in the ovary increases. Just prior to ovulation, circulating concentrations of estradiol reach a critical concentration causing a pre-ovulatory surge of LH production, ultimately leading to ovulation and release of the egg from the follicle.

After ovulation, the residual granulosa and thecal cells in the ruptured follicle proliferate and develop into the corpus luteum which is under the regulation of LH to produce progesterone. Progesterone regulates the proliferation of the endometrium or uterine lining for a potential implantation. In the absence of fertilization, the corpus luteum breaks down through a process called luteolysis. This process involves the loss of the capacity to synthesize and secrete progesterone followed by loss of luteal cells themselves. While the precise mechanisms are unknown and vary greatly among primates, in some species it has been shown to involve prostaglandins (a group of fatty

acid derivatives that mediate physiological functions through an autocrine or paracrine mechanism), specifically prostaglandin $F_{2\text{-alpha}}$ (10). As a result of the destruction of the corpus luteum, progesterone and estrogen secretions decline, and the uterine lining breaks down leading to menstruation. The loss of negative feedback by the steroids leads to a subsequent rise in FSH levels, leading to a new round of follicle development, ultimately beginning a new cycle (11).

Upon pregnancy, continued secretion of progesterone is required for the maintenance of the uterine environment for the developing embryo. This is achieved by the production of human chorionic gonadotropin (hCG) by the conceptus. The secreted hCG is structurally similar to LH and thus can rescue the corpus luteum by providing a direct growth signal for synthesis of progesterone until the placenta can take over as the dominant source of progesterone production (4).

Progesterone also plays a role in suppressing the premature termination of pregnancy by inducing quiescence of the myometrium. This is established by blocking estradiol induction of adrenergic receptors, as well decreasing the uptake of calcium and inhibiting expression of genes that encode subunits of calcium channels, all of which are required for uterine contractions (12).

1.3.3 Progestins as contraceptive agents

Progestins also have great use as contraceptive agents, wherein PR antagonists and selective PR modulators have been shown to be highly effective in blocking late follicular development, the LH surge, and ovulation. The anti-progestin mifepristone is

also used to terminate pregnancy (13). While contraceptive methods also include formulations of both progesterone and estrogen, the advent of progesterone only pills is considered safer on account of the absence of potentially carcinogenic estrogen. However in the light of recent findings that progesterone may be involved in breast cancer, the use of this steroid in contraception must proceed with caution with adequate study of the molecular activities of PR.

1.4 Other roles of progesterone

Recent evidence suggests that progesterone has several functions outside the reproductive system. PR is broadly localized in several regions of the brain and has been shown to play a major role in the central nervous system wherein it is important for the regulation of cognition, mood, inflammation, mitochondrial function, neurogenesis and regeneration, myelination and recovery from traumatic brain injury (Reviewed in (14)). A specific example of PR's role in the nervous system is seen in spinal cord injury models, wherein PR is neuroprotective in function by upregulation of brain-derived neurotrophic factor (BDNF) and choline acetyltransferase (15-17). Interestingly its functions are mediated by not only the classical PR isoforms, PR-A and PR-B, but also by the seven transmembrane domain 7TMPRβ and the membrane-associated PGRMC1, both of which will be discussed in a following section (Reviewed in (14)).

Surprisingly, serum progesterone levels in men and women are not very different, outside the luteal phase. In men, progesterone has been shown to influence

spermiogenesis, sperm capacitation/acrosome reaction and testosterone biosynthesis in the Leydig cells. (Reviewed in (18)).

1.5 The progesterone receptor

1.5.1 Progesterone receptor isoforms

Human PR has two endogenous isoforms; the 769-amino acid A-receptors (hPRA) and the 933 amino acid B form (hPRB) (Figure 1.3). The A isoform lacks 164 amino acids at the N-terminus that are present in hPRB (19). The A and B isoforms of the chicken arise by alternate initiation of translation from two in-phase AUG codons located on a single mRNA transcript (20). On the other hand in the human they are transcribed from different promoters within the same gene.(21)

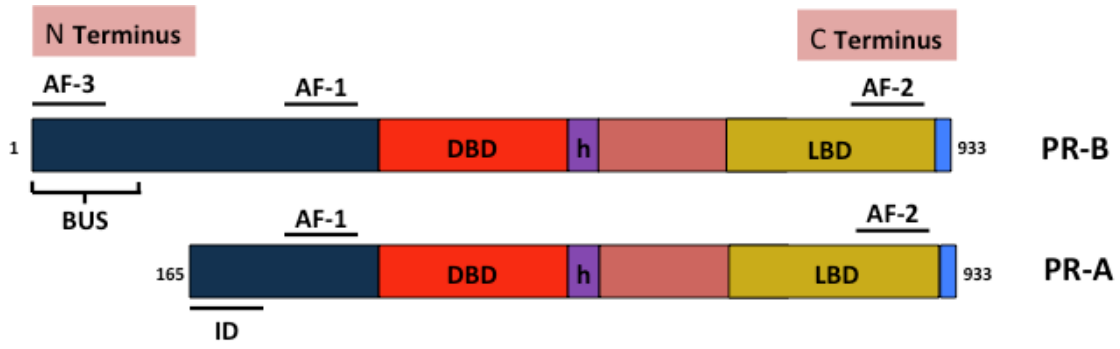


Figure 1.3: Structure of the progesterone receptor

Depiction of the various domains of the progesterone receptor isoforms PR-A and PR-B. AF- Activation function; BUS- B-upstream element; DBD- DNA binding domain; h- hinge region; LBD- Ligand binding domain; ID- Inhibitory domain

1.5.2 Structure of the progesterone receptor

Both PR-A and PR-B comprise of a modular structure with a centrally located DNA binding domain. This is flanked by a domain termed activation function 1 (AF1) on the N terminus and the ligand binding domain (LBD) on the C terminus. The LBD also contains a second activation function AF2. The activation of transcription is mediated by the two AFs. The longer PR-B isoform consists of a third activation function domain AF3 in the 1-165 amino acid region, which has been termed as the BUS region (B up-stream segment) (22) Additionally, there is a region between amino acids 638-642 termed the hinge region which is important for nuclear localization of the receptor (23).

1.5.3 Molecular activities of PR-A and PR-B

While they are similar in structure with the exception of the BUS, it has been well established that there are several differences in the agonist-induced transcriptional program for PR-A and PR-B. For example, on the MMTV promoter, transactivation by progestin-occupied PR-B was several folds higher than PR-A. There is also evidence to support a greater level of transactivation by PR-A in other conditions. For instance PR-A has been shown to be a stronger activator of transcription than PR-B on the tyrosine aminotransferase (TAT) promoter (21). Anti-progestin occupied PR-A and PR-B also exhibit functional differences. RU486-bound PR-B is able to activate transcription on a PRE containing thymidine kinase (tk) promoter, while PR-A has an inhibitory effect (19, 22). (19).

In fact studies have demonstrated that ligand-bound PR-A is a dominant negative suppressor of PR-B activity. It has been postulated that the PR-B specific AF3 domain is able to suppress the activity of an inhibitory domain (ID) contained within sequences common to PR-A and PR-B. ID has been shown to prevent PR-A from functioning as a transcriptional activator and enables it to function as a dominant repressor of PR-B (18). Further substantiating the inhibitory domain in the suppressive activities of PR-A is the observation that deletion of the N-terminal 140 amino acids (ID) from PR-A results in a receptor mutant that is functionally the same as PRB (24). An interesting observation that might serve as a potential mechanistic basis for these effects was that the residue Ser294, which lies within the ID, is preferentially phosphorylated in the context of PR-B as opposed to PR-A. It has also been shown that the N-terminal regions of the two receptors interact differently with their LBD containing carboxyl terminus. The resultant structures might lead to differential cofactor interactions, which may in turn result in differences in the transcriptional activities of the two human PR isoforms (25). For instance, both *in vivo* and *in vitro* models have shown that unlike PR-B, PR-A did not associate efficiently with the co-activators SRC-1 and GRIP1 (24).

1.5.4 Differential biology of PR-A and PR-B

Selective knockdowns of PR-A and PR-B proteins in female mice have revealed the non-overlapping biological functions carried out by each receptor. Analysis of the phenotypic effects on female reproductive function has provided evidence that the distinct transcriptional responses to PR-A and PR-B observed in cell-based transcription

assays are, indeed, representative of the biological responses elicited by these isoforms in *in vivo* models. Ablation of PR-A does not affect progesterone regulation of the mammary gland or thymus but instead results in severe abnormalities in ovarian and uterine function, leading to female infertility. More recent studies using PR-B knockout (PRBKO) mice have shown that lack of PR-B does not affect ovarian, uterine, or thymic responses to progesterone but rather results in reduced mammary ductal morphogenesis. Thus, while PR-A is responsible for the progesterone-dependent reproductive responses necessary for female fertility, PR-B is required to elicit normal proliferative responses of the mammary gland to progesterone (8).

1.6 Other PR variants

1.6.1 PR-C

Wei et al first provided evidence for a third isoform of PR named PR-C. They described a 60,000 dalton protein that was mainly localized in the cytosolic fraction of untreated T47D cells and that bound tightly to the nucleus following exposure of T47D cells to progestins (26). PR-C is N-terminally truncated, and thus lacks the first zinc finger of the DNA binding domain. However it still contains a complete hormone binding region with sequences for dimerization and nuclear localization. Studies have propagated the hypothesis that a downstream in-frame methionine within the PR-coding region can serve as a translation initiation site for the generation of a third PR-C protein (27).

However the literature surrounding this isoform remains controversial. Studies have challenged the method of isolation of this isoform and proposed that the 60-kDa protein is not a naturally occurring PR isoform (28).

1.6.2 Membrane receptors

In addition to the classical genomic mode of action, steroid hormones have been shown to trigger rapid non-genomic signaling pathways independent of any effect on transcription. These effects have been demonstrated in cells that seemingly lack the genomic progesterone receptor or gPR. The above data have raised the postulate that membrane receptors for progesterone and other steroids exist that can mediate these non-classical responses (reviewed in (29). For instance, fully developed, enucleated Xenopus laevis oocytes undergo meiotic maturation upon exposure to progesterone. Thus PR can act at an extra-nuclear site in these cells. Further evidence suggests that the site of progesterone is at or near the cell surface and is mediated by ionic changes within the oocyte (30).

Recent times have seen the emergence of two types of membrane proteins that are distinct from the classical progesterone nuclear receptor-i) progesterone membrane receptors (mPRs) and ii) progesterone receptor membrane component one (PGMRC1 [also known as progesterone receptor membrane component 1, PGRMC1]). The mPRs in humans comprise at least three subtypes, α, β and γ and belong to the seven-transmembrane progesterone adiponectin Q receptor (PAQR) family. In mammals, the mPRαs have been implicated in progesterone regulation of uterine function in humans

and GnRH secretion in rodents (31). PGRMC1, predominantly found in the endoplasmic reticulum has been linked to acrosome reaction (32) and to the antiapoptotic action of progesterone in granulosa cells (33).

However, conflicting evidence in regard to membrane receptors continues to make them a debated phenomenon. A study was able to isolate a homologue of human PR in Xenopus which manifested transcription activity similar to human PR. The Xenopus progesterone receptor (xPR) was shown to be excluded from the nucleus and reside in the cytoplasm, as opposed to being associated with the membrane (34). Studies done on the human spermatozoa are widely cited as evidence for mPRs since it has been shown to mediate progesterone-induced acrosome reactions in sperm (35). However, this finding has come into question given the discovery that spermatozoa do in fact possess small amounts of gPR (36).

1.7 Mechanisms of PR action

Briefly, in a hormone-free environment, the progesterone receptor is maintained in an active form in the cytoplasm as a complex with heat shock proteins and immunophilins. Upon ligand binding, the receptor undergoes a conformation change leading to its dissociation from the complex, thereby enabling it to dimerize and enter the nucleus. The receptor can now interact with available cofactors to recruit transcription machinery and enable transcription of target genes (Figure 1.4).

In the native hormone-free state the receptor exists in a large multiprotein complex that was termed the 9S form. Toft et al discovered that they could reconstitute a

receptor heat-shock protein complex upon incubation of immune-purified chick progesterone receptor with rabbit reticulocyte lysate. The chicken PR was found in association with rabbit heat shock proteins hsp90 and hsp70 in a temperature-dependent manner (37). They were able to demonstrate that the steroid-bound receptor did not associate with the heat shock proteins, thus confirming that agonist binding can release the receptor from the heat shock protein complex. By a general approach of using a specific monoclonal antibody to immunoadsorb a steroid receptor or heat shock protein and then determine the other proteins that are co-immunoadsorbed, various other proteins that are part of the inactive PR-Hsp complex were uncovered. Hsp90, Hsp 56, FKBP51, FKBP52, Cyp-40, p60, p23 are other proteins have been identified as part of the bound 9S receptor complex (38) (39, 40).

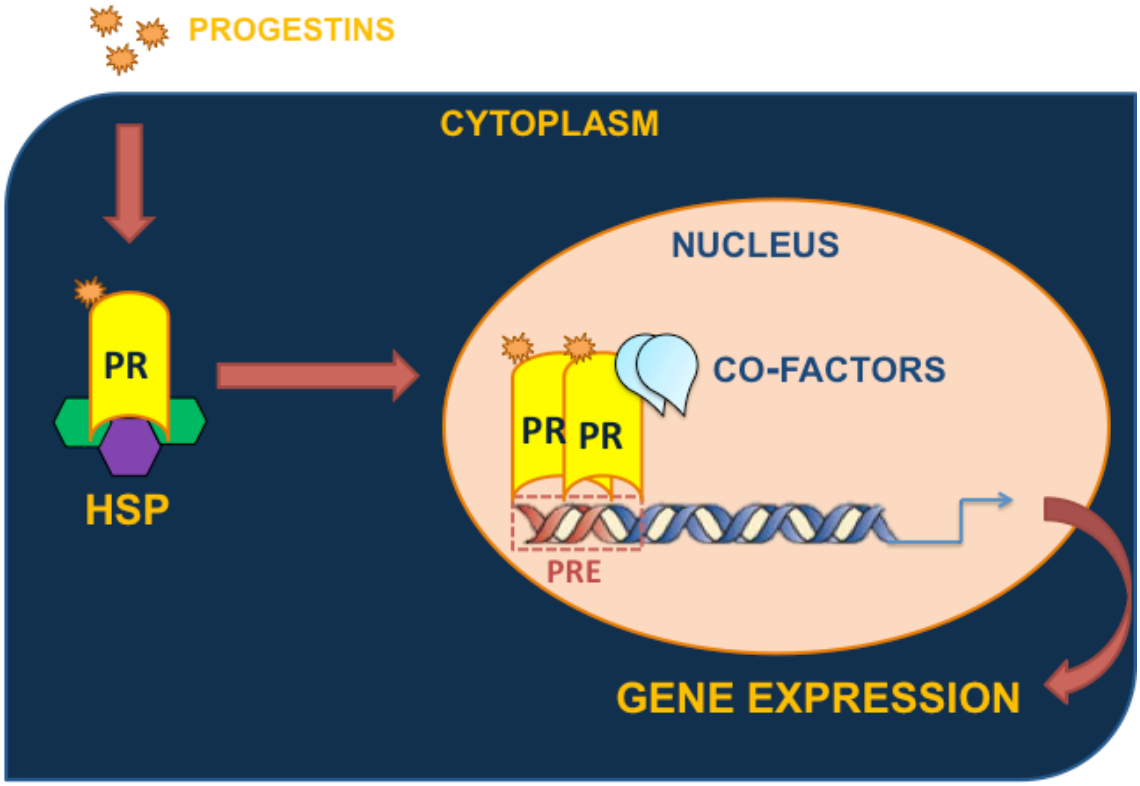


Figure 1.4: Model depicting the molecular mechanism of PR transcriptional activation in the cell.

1.7.1 DNA binding

Upon ligand binding, receptor dimers translocate to the nucleus and bind to specific DNA elements found in the regulatory regions of target genes. This process is also accelerated by the interaction with co-factors that facilitate the opening of chromatin and recruitment of the transcription machinery. The consensus steroid response element identified for several nuclear receptors including the progesterone receptor is the palindromic sequence with 5′-TGTTCT-3′ as the half-site, separated by a three base-pair spacer. It has been shown that these DNA response elements are not merely binding regions for steroid receptors. These regions can induce conformational changes upon

receptor binding that can hence enable or disable binding to certain co-factors. (Reviewed in (41)). Studies have also calculated a position weight matrix to predict PR binding sites in the genome via extensive analysis of available data (14).

The typical steroid receptor DNA binding domain consists of a well-conserved domain of 66 residues, and a variable C-terminal extension of approximately 40 amino acids (aa 633-656). The C-terminal extension is in fact also known as the hinge region that was mentioned previously. This region is capable of binding regulatory partners as well as DNA; hence it is also termed as the carboxy-terminal extension of CTE (42). The core is a well-conserved region consisting of two α-helices and two zinc modules (43, 44). The zinc modules, also known as the zinc finger domains, consist of two zinc ions, whose actions are co-ordinated through a tetrahedral arrangement of four cysteine sulphurs. (45). This is essential for DNA binding as the metal co-ordination has been shown to be required for the receptor to adopt its active conformation. Three residues at the beginning of the DNA recognition helix 1 (or P box) play an important role in recognizing specific steroid receptor element sequences, while the C-terminal zinc-binding module contains a D box which is important for DNA binding-dependent dimerization of the DBD (46).

Interestingly, the DNA-bound PR structure exhibited a compressed minor groove (47). While other steroid receptor DBD–DNA structures have also exhibited a compressed minor groove width, it has been claimed to be most distorted in the PR–DNA complex (Reviewed in (42)). Receptor-induced distortion and bending of DNA has been

also been postulated as important for different cofactor interactions or interactions with distal enhancers and protein complexes (42).

1.7.2 Co-activator functions

Upon hormone binding and receptor activation, the receptor dimer can enter the nucleus and affect transcription by collaboration with available co-activators that assist PR in the recruitment of transcription machinery and enhance gene expression (Figure 1.5). Evidence indicates that the AF-2 domain is responsible for the agonist-bound receptor's ability to recruit co-factors. The AF-2 region on the steroid receptor is able to interact with an LXXLL motif (L- Leucine, X- Any amino acid) that is found on the surface of certain co-factors such as RIP-140, SRC-1 and CBP(48).

To date a plethora of close to 200 nuclear receptor co-regulators have been identified that play different roles in various cellular processes (49). In fact it has been suggested that multiple co-factors may associate with the receptor in a temporal manner in order to regulate the expression of a particular gene or gene set (49). These co-activators can exist in individual or heterologous complexes and can be modified by post-translational modifications such as phosphorylation or acetylation that can thus modify its affinity for the receptor complex (50, 51). These co-factors thus present another way to regulate nuclear receptor actions besides ligand binding. Other than their importance in the initiation of transcription, nuclear receptor co-factors have also been known to be involved in alternate RNA splicing and termination of regulated transcription, among other things (52, 53).

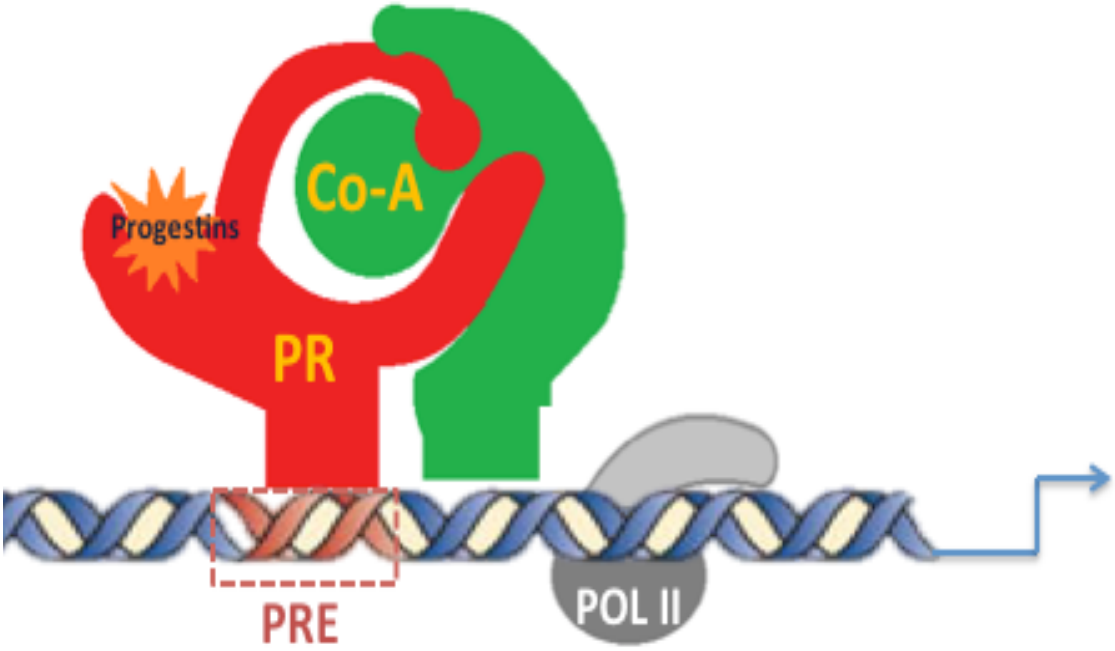


Figure 1.5: PR-DNA binding model

Model depicting DNA binding and recruitment of co-activators and pol II containing transcription machinery to the DNA to activate gene expression. PRE- progesterone response element; Pol II- RNA polymerase II; Co-A- Coactivator.

SRC-1 (NCOA-1) was the first SRC/p160 co-activator shown to interact with ligand-bound PR (54, 55), following which considerable studies have emerged that explore the importance of the co-factors SRC 1-3 in PR signaling. For instance, PR preferentially recruits histone acetyltransferase (HAT) activity containing co-factors SRC-1, SRC-3, and CBP, but not SRC-2 or pCAF, leading to specific histone modification of a chromosomal mouse mammary tumor virus promoter (56). This highlights differential recruitment of co-activators by nuclear receptors as a mechanism

to mediate specific gene regulation by steroids. The SRC-1 null mice demonstrate partial hormone resistance in progesterone target tissues, such as mammary gland and uterus, thus establishing the importance of SRC-1 for the physiological actions of progesterone (57). In addition, ASC2, TIP60, GT198 and SNURF are other examples of proteins that have been shown to be PR co-activators (Reviewed in (58)).

1.8 Non-genomic functions and cross-talk with other signaling pathways

Although the classical genomic pathway of PR activation of target-gene transcription is well established, PR has also been shown to function via non-genomic signaling pathways that do not require its role as a transcription factor. A prominent example of the above phenomenon is that the polyproline motif in the N-terminal of PR has been shown to interact with the SH3 domain of c-Src tyrosine kinase to mediate progestin-dependent downstream MAPK signaling (59) (Figure 1.6). The clinical relevance of PR-mediated Src signaling is high considering the strong positive correlation of Src expression with breast cancer. Besides MAPK signaling, Proietti et al have shown that progestins can induce Stat3, Jak1 and Jak2 phosphorylation in *in vitro* models of breast cancer. Moreover, progestins were shown to positively regulate Stat3 transcriptional activation, which was in turn necessary for progestin stimulation of both *in vitro* and *in vivo* breast cancer growth (60). Progestins have also been shown to induce cell proliferation through up-regulation of cyclin D1 expression via phosphatidylinositol 3-kinase/Akt/nuclear factor-kappa B pathway in human breast cancer cells (61).

In vivo studies have demonstrated cross talk of PR with neurotransmitters wherein PR-mediated reproductive functions in rats and mice rely on dopamine-induced activation of PR (62, 63). A dopamine receptor stimulant and a D1 receptor agonist mimicked the effects of progesterone in enabling sexual behavior in female rats. This effect was inhibited by both progesterone receptor and D1 receptor antagonists, as well as by antisense oligonucleotides to the progesterone receptor. ER and PR have also been known to have overlapping pathways, wherein ER is required for several PR-mediated biological functions. On the other hand PR can also suppress the ER-induced proliferation in the uterus (64).

Convergent pathways for PR and other hormones and growth factors are highlighted by the fact that there is now strong evidence that early pregnancy protects against onset of breast cancer (65, 66). Pregnancy induced levels of estrogen and progesterone can lead to persistent changes such as sustained expression of nuclear p53 in the molecular landscape of mammary glands that are resistant to tumorigenesis (67, 68).

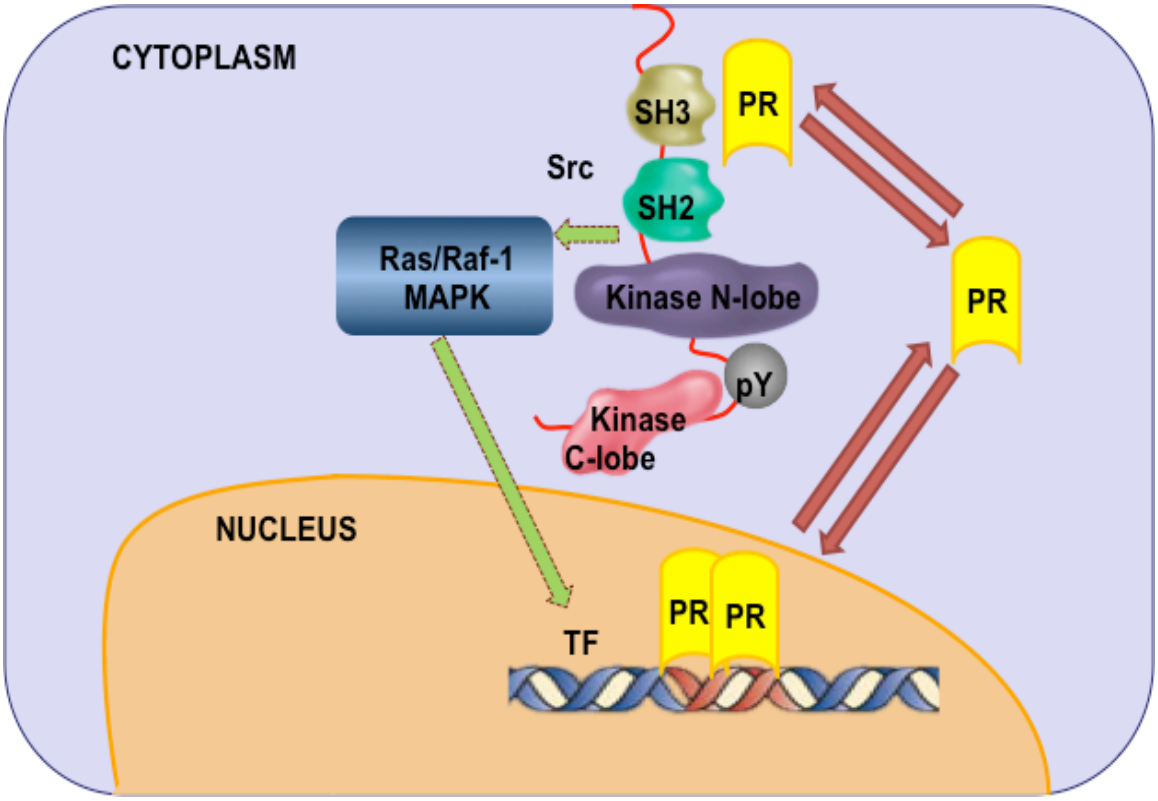


Figure 1.6: Non-genomic actions of PR

Model depicting the rapid cytoplasmic signaling by PR via the Src/Ras/Raf-1/MAPK pathway. A polyproline motif on the N terminus of PR can interact with the SH3 domain of Src, thus enabling it to activate downstream signaling to activate gene transcription. TF- Transcription factor. Adapted from (69)

1.9 Post-translational modifications of PR

Apart from genomic regulatory regions and co-activators in the cellular environment, PR activity is also modified by post-translational modifications that include phosphorylation, ubiquitination, SUMOylation and acetylation. The signaling pathways that govern these alterations can modify the stability, hormone sensitivity and nuclear localization of both agonist-bound and agonist-free receptors.

At least 14 phosphorylation sites have been identified on PR-B, with Ser102, 294 and 345 being ligand-dependent sites. Examples of ligand-dependent kinases include CDK2, MAPK, PKA and tetradecanoyl 12-phorbol 12-acetate (PKC activator) (Reviewed in (70)).

Ubiquitination, which consists of the binding of a 76 amino acid moiety to lysine residues on substrates, targets the altered proteins to varied fates such as internalization and activation or proteosomal degradation (71). PR activity is stimulated by the ubiquitin ligases hRP51 (72) and E6-AP (73), while SRC-1 function as a co-activator requires the ubiquitin-conjugating enzyme UBCH7 (74). The most common effect of PR polyubiquitination however is ligand-dependent downregulation of the receptor (75).

PR is also subject to regulation by small ubiquitin-related modifiers or SUMO. PRs have one ligand-dependent SUMO binding consensus sequence at lysine (K) 388 in the N-terminal domain. PR SUMOylation manifests a suppressive effect on transcription (76, 77)

PR is also subject to acetylation as a post-translational modification. Daniel et al have shown that PR is acetylated at a conserved KxKK motif located at amino acids 638-

641 in the hinge region, and this modification has a transcription inhibitory effect (78). Disruption of the acetylation motif results in receptors that require at least 4hrs of ligand treatment to translocate to the nucleus, in contrast to 15 min required for wild-type PR to translocate to nucleus in the presence of ligand (78)

1.10 Relevance of PR in breast cancer

Breast cancer is one of the leading causes of death for women with an estimated 232,340 new cases of invasive breast cancer expected to be diagnosed among women in the US during 2013 and about 2,240 new cases are expected in men (*"Cancer Facts and Figures 2013". American Cancer Society, Atlanta, Ga; 2013)*.

The majority of breast tumors, approximately 75%, express the estrogen receptor (ER), and more than half of these cancers also express PR (79). Not surprisingly therefore, interventions that block the production and/ or the actions of estrogens are frontline therapies for breast cancer. While the mitogenic effects of estrogen in breast cancer are well understood, the role of progestins in breast cancer progression is less clear. Progestins are often used in combination with estrogen for Hormone Replacement Therapy (HRT) in post-menopausal women due to their ability to inhibit the unchecked proliferative actions of ER in the uterus (80). The Women's Health Initiative (WHI), which was undertaken to evaluate the risks and benefits of the combined HRT was stopped prematurely because it was noted that women taking the combined therapy were at greater risk of developing invasive breast cancer than women receiving estrogen alone (81). Two randomized clinical trials begun in the 1990s in Scandinavia investigated the

safety of HRT with any new event of breast cancer as the end point. Similar to the WHI trial, this longitudinal study (named HABITS-hormonal replacement therapy after breast cancer--is it safe?) was also suspended before completion because it was decided that HRT posed an unacceptable risk of breast cancer to exposed women (82). A thorough analysis of epidemiological data from 54 studies from 25 countries to evaluate breast cancer risk and the use of hormonal contraceptives also showed some evidence that use of progestin containing contraceptive use may contribute to breast cancer risk (83).

The aforementioned data are strong indicators that progestins may contribute to breast cancer progression, which brings to the forefront the question of whether antiprogestins could be useful anti hormonal interventions. However the available data on the use of progesterone antagonists is not definitive. Initial studies of RU486 in animal models demonstrated that RU486 was as effective as the antiestrogen tamoxifen at inhibiting breast tumor growth (84). However, this result did not translate to clinical studies. In a series of small clinical trials RU486 was shown to have only a moderate response rate in patients with metastatic, tamoxifen-resistant cancer (85). However, the incertitude of this study may result from the fact that the patients were enrolled independent of PR and ER status. Similarly, a study that was undertaken to evaluate the PR antagonist Onapristone (ZK 98 299) as a first-line therapy against breast cancer was unable to continue recruitment of patients owing to the fact that some patients treated with this drug developed liver function abnormalities. Although the trial was carried out with the patients enrolled and showed positive results, it was rendered statistically insignificant (86).

Taken together, the above data presents us with enough rationalization to invest scientific resources in the investigation of the role of PR in breast cancer initiation and progression, with the ultimate goal of harnessing this information for anti-PR therapy in the clinic.

1.11 The cell cycle and PR.

The cell cycle is a complex and tightly regulated process, which involves the carefully coordinated actions of several proteins- mainly the Cyclin dependent kinases (CDKs) and cyclin proteins (Reviewed in (87). The cyclins, so named due to their cyclical expression throughout the cycle, are the proteins that govern the phase of the cell cycle based on their expression (88, 89). The CDK proteins are serine/threonine kinases that are activated by the cyclin proteins (90) and push the cell through various check-points by the phosphorylation of target proteins. Once their target(s) has been stimulated to trigger further downstream gene expression, they are in turn subject to regulation by proteins belonging to the p21 and p16 families which serve to attenuate their activity (91-93).

Briefly, upon stimulation of a quiescent cell in G_0 by growth factors, the cell enters G1 (gap 1) with the expression of Cyclin D1 which promotes the formation of Cyclin D1-Cdk 4/6 complexes, which in turn phosphorylate its downstream target Rb. This is turn leads to further phosphorylation of Rb by the Cyclin E/Cdk 2 complex, ultimately leading to the transition into S (synthesis) phase. The action of Cyclin A-Cdk 2 complexes permit the cell to move into the G2 (gap 2) phase, following which Cyclin B-

Cdk 1 complexes enable the cell to complete mitosis (M phase) and thus divide (Reviewed in (87)).

Progestins have been shown to modulate cellular proliferation *in vitro* as well as in animal models of breast cancer (94). In PR+/ER+ T47D cells, synthetic progestin treatment results in an acceleration of cells through one passage of the cell cycle followed by arrest in early G1. It has been postulated that induction of progestin responsive genes such as Cyclin D1 and cMyc followed by the downstream phosphorylation of Rb and release of E2F, which induces the transcription of cell-cycle genes, enables G1-S transition (95-97). On the other hand, the subsequent cell cycle arrest correlates with a decrease in Cyclins D and E and downregulation and dephosphorylation of Rb, as well as induction of cyclin-dependent kinase (CDK) inhibitors p21 and p27(Kip1) and their actions on Cyclin D-cdk4 and Cyclin E-cdk2 complexes (98, 99). The inhibition of growth by PR antagonists has been relegated to increased p21 expression and synchronous inhibition of CDK activity. The importance of Cyclin D1 in anti-progestin mediated growth inhibition was demonstrated by the reversal of the inhibition of CDK activity upon ectopic expression of Cyclin D1 (100). While the above studies point to several molecular targets that contribute to PR's effects on proliferation, more work is required to elaborate on the mechanisms of PR's proliferative and anti-proliferative mechanisms.

Overall, all of the above studies highlight the fact that PR clearly plays a complex and major role in breast cancer prognosis. Given that over half of all breast cancers express PR, there is an unmet potential for the use of this receptor as a drug target to

combat tumor growth. However, until the molecular participants in PR-mediated regulation in breast cancer are completely understood, appropriate drugs to target specific PR actions in breast cancer cannot be conceived. The knowledge gained from the projects described in this thesis will aid in further understanding PR biology in breast cancer and the identification of selective PRMs (progesterone receptor modulators) that can be used to inhibit specific PR actions and therefore breast cancer progression.

2 Genomic regulation of Cyclin D1 by progestins

2.1 Introduction

Within the confines of normal physiology, the steroid hormone progesterone plays a key role in the development and differentiation of the female reproductive system. The biological functions of this hormone are mediated through two progesterone receptor isoforms- PR-A and PR-B which in the human are transcribed from different promoters within the same gene. In the absence of ligand, the receptors are sequestered in an inactive state in the cytoplasm by heat shock proteins. Upon ligand binding, the receptor undergoes a conformational change that releases it from the inhibitory heat shock protein complex and enables receptor dimerization. The receptor dimer enters the nucleus and positively or negatively regulates transcription by binding either to (a) specific progesterone response elements (PREs) within target genes or (b) transcription factors already associated with DNA (tethering). In this manner, PR can initiate a cascade of events that leads to the induction of both primary and secondary response genes that alter cellular phenotype (58).

In addition to their activity as transcription factors the PRs can regulate target gene transcription in an indirect manner through their ability to modulate signaling pathways that impinge on transcription factors located at target genes. For example, a polyproline motif within the amino terminus of PR has been shown to interact with the SH3 domain of the c-Src protein and in doing so it initiates a signaling cascade that results in the rapid activation of the mitogen activated protein kinase (MAPK) (101).

These rapid actions of PR/progestins have also been shown to be important in the activation of the phosphoinositol 3-kinase/Akt pathways as well as pathways regulated by the Janus family of tyrosine kinases (JAK) and signal transducer and activator of transcription (STAT) (60, 61). It is worth noting that the activity of PR in these pathways has been demonstrated *in vitro* although confirmation of their importance in the physiological or pathophysiological actions of progestins remains to be demonstrated.

2.1.1 Phosphorylation of the Rb protein by progestins

Most models describing PR action highlight the ability of the DNA bound receptor to nucleate the assembly of large proteins complexes within the regulatory regions of genes that alter chromatin structure and influence PolII transcription. However, in reality the transcriptional activities of PR are much more complex and are more appropriately classified as multi-modal. In a recent publication from our laboratory we reported that progestins upregulate the expression of E2F1 in a multimodal fashion (102). Specifically, it was demonstrated that PR (i) binds directly to the E2F1 promoter, and (ii) induces the expression of KLF15 which itself binds to the E2F1 promoter. Further, it was observed that addition of progestins led to a hyper-phosphorylation of the retinoblastoma (Rb) protein, and the subsequent release of active E2F1 which in turn bound to its own promoter inducing further expression of E2F1. (103). Whereas our studies were informative with respect to the events that occurred on the E2F1 promoter, the mechanism(s) by which PR activation resulted in Rb phosphorylation remained an open question.

The retinoblastoma protein is a tumor suppressor that functions as a negative modulator of the expression of several key cell cycle genes such as E2F1, E2F2, Cyclin E etc. In resting (G0) cells the primary function of Rb is to suppress the activity of E2F1 by sequestering the protein and preventing it from associating with the regulatory regions of its target genes. Upon exposure to a mitogenic stimulus the Rb protein undergoes phosphorylation; an activity that is incompatible with E2F1 binding. In this manner the phosphorylation of Rb enables the rapid association of E2F1 with its target genes. Phosphorylation of Rb is carried out by cyclin dependent kinases CDK4/6 when complexed with Cyclin D1, and/or Cdk2-Cyclin E complexes (104). There are several studies which suggest that MAPK activation by PR results in increased expression of Cyclin D1 expression albeit by an unknown mechanism. The latter findings remain somewhat controversial and it is clear that further exploration of the PR/Rb/E2F1 axis is warranted.

Regardless, given its primacy in the cell cycle, it is no surprise that dysregulation of this pathway has been shown to be important in the genesis of many cancers. Notably, the expression of Rb itself is abnormal in 20-30% of breast cancers (105-107). Overexpression of the Rb/E2F target gene set, which is indicative of Rb functional inactivation, is also associated with disease progression in ER positive breast cancer patients that have developed tamoxifen resistance (108). Together these data highlight the need to define the mechanisms by which PR impacts Rb phosphorylation and how this pathway can be exploited in the pharmacotherapy of breast cancer and meningiomas where increased expression and activity of PR is noted. Thus the primary goal of this

study was to define the biochemical links between PR activation and Rb phosphorylation; a pathway of importance in breast cancer.

2.2 Results

2.2.1 The T47D cell line to study the role of PR in breast cancer

In most cell models of luminal breast cancer estrogens are required to induce the expression of PR. In addition estrogens, through their cognate estrogen receptor (ER) also increase the expression of Cyclin D1 in breast cancer cells. Thus, it has been exceedingly difficult to uncouple the effects of estrogen and progestins on the regulation of Cyclin D1. However, in T47D:A18 cells the expression of PR does not require ER affording us the opportunity to "isolate" PR signaling and evaluate the specific impact of progestins on Cyclin D1 expression. Thus, we chose to use T47D:A18 as the primary model system to define the molecular mechanisms by which PR impacts Cylin D1 expression (109, 110).

2.2.2 The MAPK pathway is not required for progestin-mediated induction of Rb hyper-phosphorylation in breast cancer cells

In a previous study published by our laboratory we reported the observation that progestin mediated induction of the expression and subsequent hyper-phosphorylation of Rb was partially inhibited by the MEK 1/2 inhibitor U0126. Although this finding was not followed up on at that time it did suggest that Erk 1/2 activation was required for PR-mediated Rb phosphorylation. Interestingly, for this and most other studies U0126 was used at a concentration of 10uM. However, it has now been established that at this concentration U0126 has substantial off-target activities on Cdk4/6 kinase activity raising

doubt as to the use of this compound to implicate MAPK signaling (111). We confirmed using P-Erk as an endpoint that complete inhibition of MEK1/2 could not be achieved with doses of U0126 of less than 10uM (Figure 2.1). These important data suggested that in addition to evaluating the potential impact of MAPK on Rb phosphorylation that we had also to consider the involvement of CDK 4/6 in this activity.

To address this issue we examined the effect of two additional MEK inhibitors on progesterone induced Rb phosphorylation. As can be seen in Figure 2.1 neither AZD6244 nor PD184161, drugs that do not inhibit Cdk4/6 at the doses used in our studies (100nM), have any effect on basal or progestin induced phosphorylation of Rb. It was concluded therefore that the MEK/ERK pathway was not required for progesterone mediated induction of Rb phosphorylation and that studies that used U0126 to implicate this pathway need to be reevaluated. Indeed, the data was compatible with the idea that the activity of U0126 on Rb phosphorylation maybe related to its ability to inhibit CDK4/6; a result that led us to reconsider that the impact of progestins on Rb phosphorylation may be related to their ability to activate CDK4/6 activity. It was for this reason that we begun to explore the possibility that the CDK 4/6 activator Cyclin D1 expression may be regulated by PR/progestins.

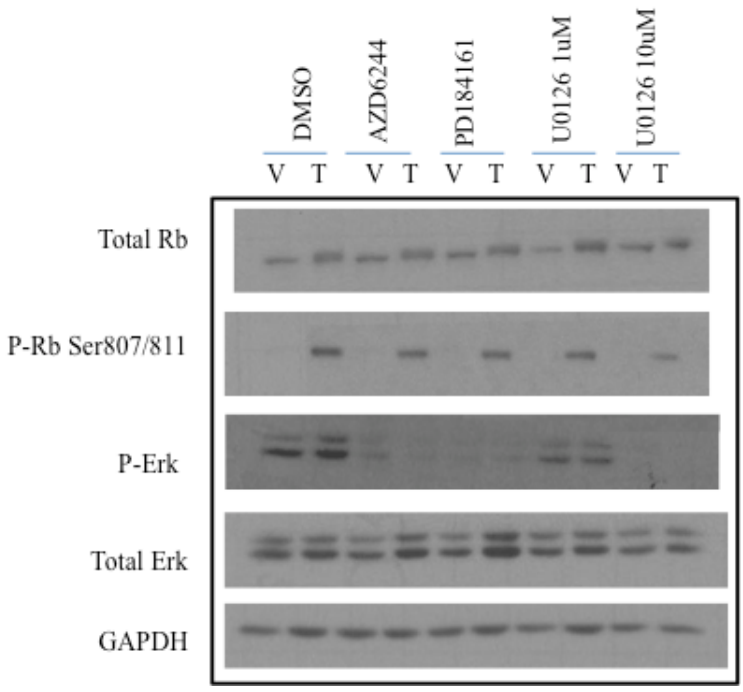


Figure 2.1: Progestin-mediated induction of Rb hyper-phosphorylation does not require activation of the MAPK pathway.

Synchronized T47D:A18 cells were pre-treated with DMSO or the MEK inhibitors AZD6244 (100nM), PD184161(100nM), U0126 (1uM) or U0126 (10uM) 30min prior to the addition of Vehicle (V) or 100pM R5020 (T) for 18hr. After treatment, whole-cell extracts were prepared and immunoblotted for total Rb, Rb phosphorylated on Ser807/811 (p-Rb Ser 807/811), P-Erk, GAPDH or Total Erk (Erk 1/2). GAPDH and Total Erk were used as a loading control. A representative blot is shown (*n =3)*.

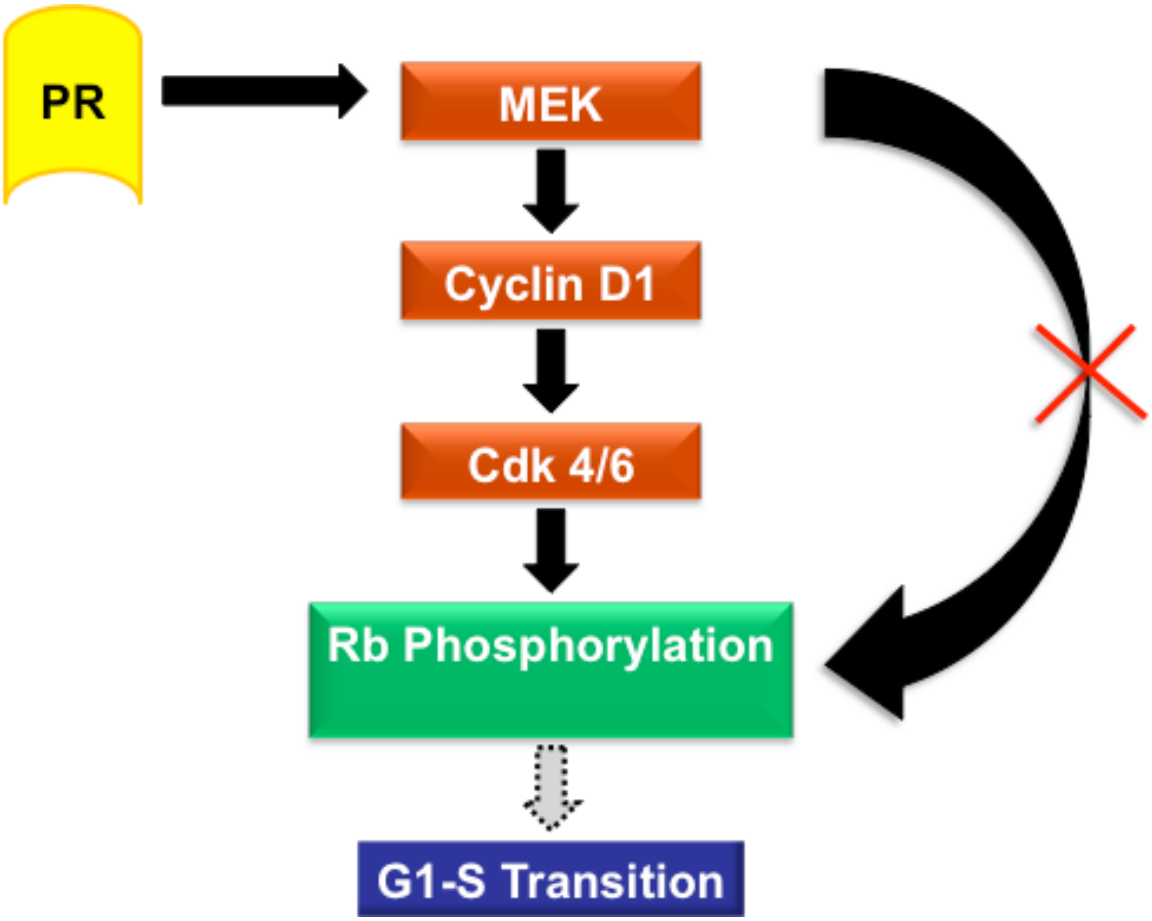


Figure 2.2: Model depicting alternate pathways for progestin mediated Rb phosphorylation.

2.2.3 Cyclin D1 expression is responsive to progestin treatment in cellular models of breast cancer.

It has been established that phosphorylation of Rb is accomplished primarily by Cdk4 subsequent to its activation by Cyclin D1. Further, it has been demonstrated by others that Cyclin D1 expression in breast cancer cells can be induced by progestins (101) (112). However, the mechanism proposed by Boonyaratanakornkit et al suggested that upon activation the polyproline motif within the N-terminal domain of PR interacts with the SH3 domain of Src, activating the Src/Ras/MAPK pathway, resulting in the induction

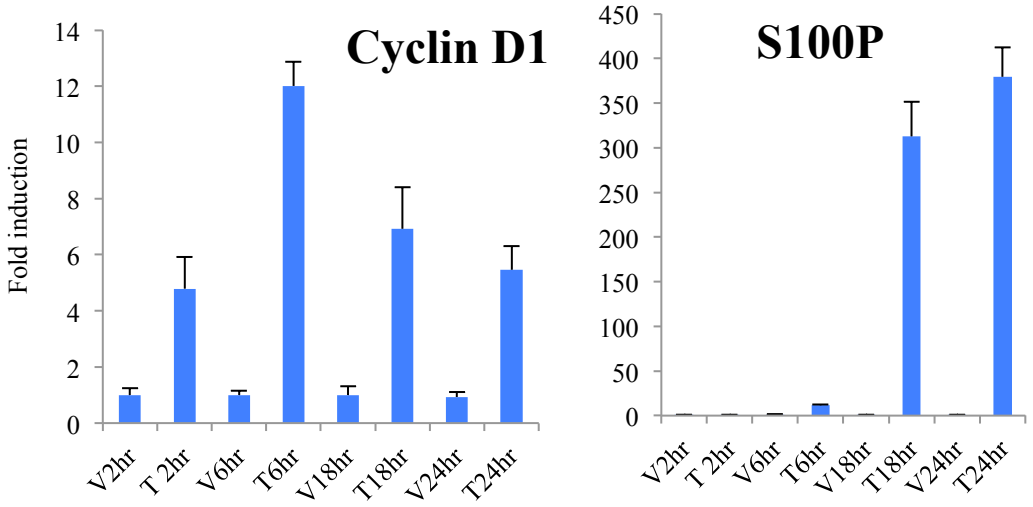
of the expression of Cyclin D1 (101). Unfortunately, the major piece of data that linked the activation of the MAPK pathway and Cyclin D1 expression was the observation that U0126 could block the action of PR. For the reasons stated above we now believe that the conclusions of this study need to be reconsidered. Moreover, comparison of R5020 induction of E2F1 in the PR negative T47D:C42 cells that stably express wild type PR-B or a mutant form of PR-B (PR-B mPro) that can no longer interact with the Src family kinases and downstream MAPK pathway, showed no difference (102). Since E2F1 is a downstream target of Cyclin D1 via Rb phosphorylation, this data implies that Cyclin D1 induction should also remain unaffected. Along the same lines, studies carried out by a previous graduate student Hilary Eaton (unpublished data) using the Src kinase inhibitor SU6656 have shown that R5020 mediated Cyclin D1 induction at 6hr is not significantly affected. This implies that disabling PR activation of the Src/Ras/MAPK does not significantly affect Cyclin D1 induction. Thus, the above data highlight the need to explore the possibility of a more direct effect of PR on Cyclin D1 expression.

Several other mechanisms for PR induction of Cyclin D1 have been proposed. Quiles et al show that this function requires both the DNA binding domain and the estrogen receptor interacting domain of PR to be intact, the latter of which enables PR to activate the MAPK cytoplasmic signaling pathway through binding to ERα. In addition, it also binds to two regions on the distant Cyclin D1 promoter (112). Two other papers also showed that PR is recruited to a promoter region of Cyclin D1 via a tethering mechanism. Cicatiello et al claimed that it was tethered to an AP-1 complex while Beguelin et al suggested that it was through an Erb-B2/Stat3 complex (113, 114).

However we did not observe PR recruitment to these regions in our hands (data not shown).

To explore potential genomic mechanisms underlying PR mediated induction of Cyclin D1 expression we collected mRNA and proteins from synchronized T47D cells treated with R5020 for 2, 6, 18 and 24 hours. It was observed that the expression of Cyclin D1 mRNA was detectable as early as 2hrs following treatment, peaking at 6hrs with a dramatic downregulation apparent by 18hrs continuing to 24hrs (Figure 2.3 A). This induction pattern was also observed at the level of Cyclin D1 protein. (Figure 2.3 B). The kinetics of the mRNA induction were compatible with a primary effect of PR on Cyclin D1 expression; a possibility we explored in our continued studies.

A)



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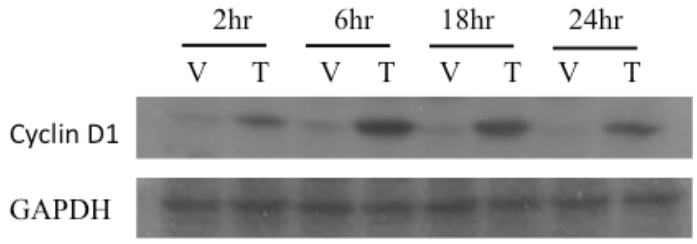


Figure 2.3: The expression of Cyclin D1 mRNA and protein are induced by progestins.

Synchronized T47D:A18 cells were treated with Vehicle (V) or 100pM R5020 for the time-points indicated. A) After treatment, cells were lysed and RNA was isolated and Cyclin D1 mRNA levels were quantified using qPCR and normalized to the expression of the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). The expression of a well chaarcaterized PR-target gene S100P was evaluated in these studies for comparative purposes. B) After treatment, whole cell extracts were prepared and subjected to immunoblotting for Cyclin D1 and GAPDH (loading control).

2.2.4 Cyclin D1 is required for progestin-mediated phosphorylation of Rb.

In order to establish whether Cyclin D1 is necessary for progestin-mediated Rb phosphorylation, we utilized siRNA technology to knockdown Cyclin D1 mRNA and protein levels in synchronized T47D cells. As shown in Figure 2.4, quantitative knockdown of Cyclin D1 mRNA and protein was achieved in these cells and this attenuated the ability of progestins to induce Cyclin D1 levels. Importantly, progestin mediated Rb phosphorylation was also attenuated by Cyclin D1 knockdown.

Inhibition of the expression of E2F1 mRNA, and the absence of a change in FKBP expression (a classical PR-target gene), confirmed the functional significance of inhibiting progestin-mediated induction of Cyclin D1 expression and Rb phosphorylation and highlighted the specificity of this response. Thus, these data prompted us to consider that the actions of PR on the phosphorylation status of Rb are dependent on PR upregulation of the expression of Cyclin D1.

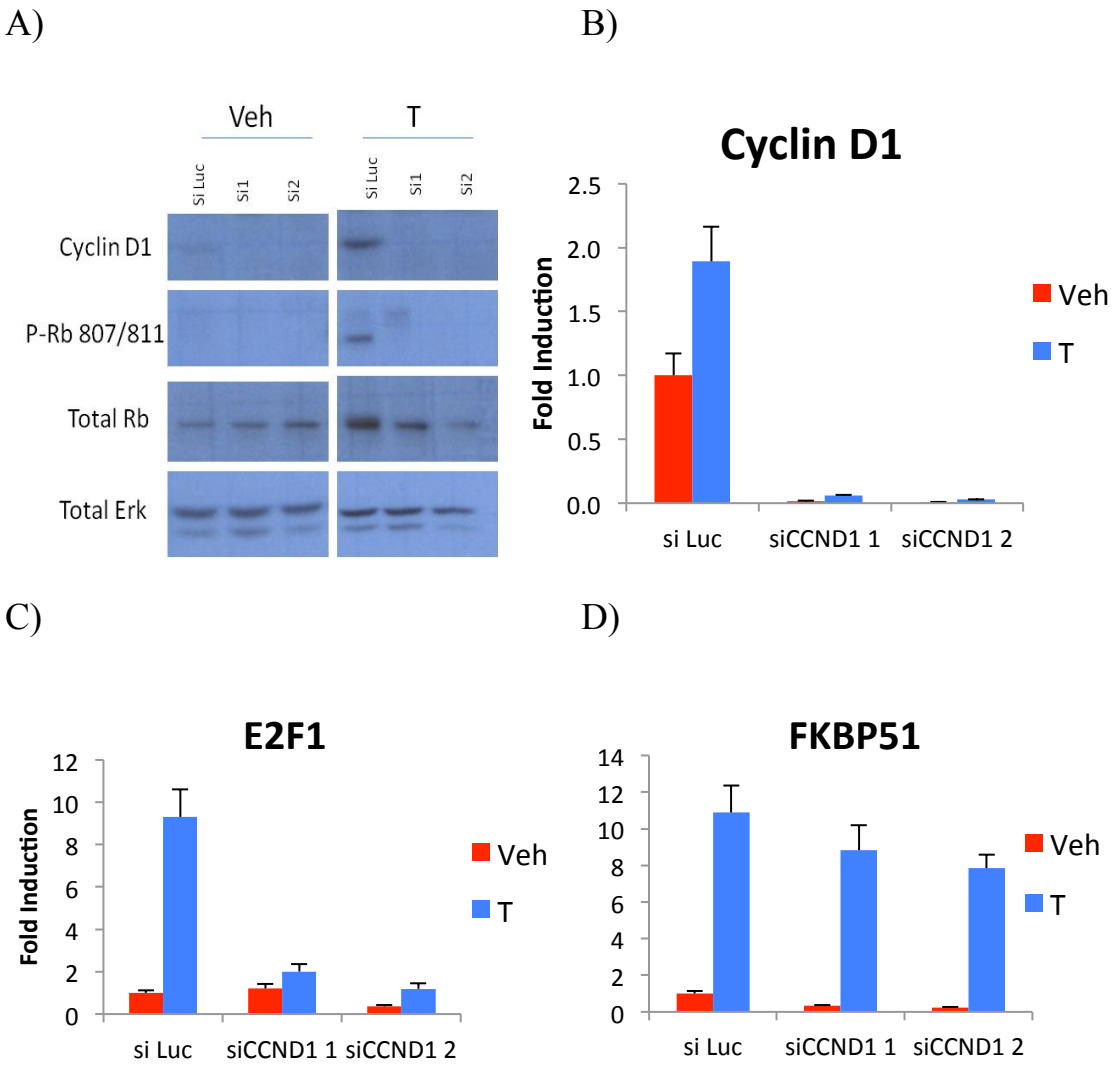


Figure 2.4: Cyclin D1 is required for progestin-mediated Rb phosphorylation.

Synchronized T47D:A18 cells were transfected with 2 different siRNA sequences to Cyclin D1. Cells were treated with Vehicle (Veh) or 100pM R5020 (T) for 18hr. A) Whole cell extracts were prepared and immunoblotted for Cyclin D1, Total Rb, p-Rb 807/811 or GAPDH (loading control). B) Cyclin D1 mRNA levels were quantified using qPCR and normalized to the expression of the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). C and D) The expression of E2F1 and FKBP mRNA in response to R5020 was assessed in cells following CyclinD1 knockdown.

2.2.5 Cyclin D1 is a direct target of PR in cellular models of breast cancer

The rapid induction of Cyclin D1 mRNA expression following the addition of progestins to cells suggested the possibility that this gene was a primary target of PR. Consistent with this hypothesis we demonstrated that pre-treatment of synchronized T47D cells with the transcription inhibitor actinomycin D inhibited PR-mediated Cyclin D1 induction (data not shown). Further it was also demonstrated that the induction of Cyclin D1 mRNA expression was not attenuated by the translational inhibitor cycloheximide, implying that it was a direct target of PR (Figure 2.5). As expected, given that PR-mediated induction of KLF15 is required for its expression, it was observed that E2F1 mRNA expression was completely inhibited by cycloheximide.

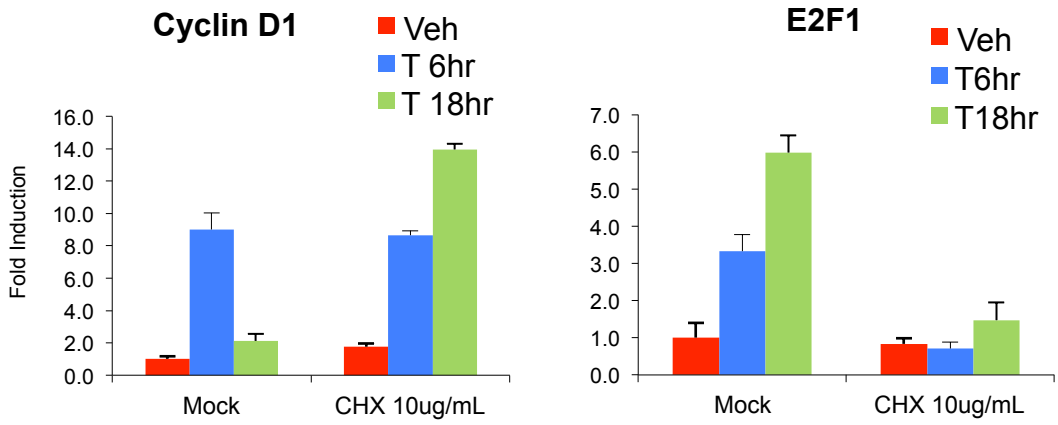


Figure 2.5: Cyclin D1 is a direct target of PR.

Synchronized T47D:A18 cells were pre-treated with the translation inhibitor cycloheximide (CHX) for 1hr, followed by treatment with Vehicle (Veh) or 100pM R5020 (T) for the time points indicated. After treatment, cells were lysed and RNA was isolated and Cyclin D1 and E2F1 mRNA levels were quantified using qPCR and normalized to the expression of the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). E2F1 is shown here as a control for cycloheximide treatment as it has been previously shown that maximal induction of E2F1 by progestins requires the induction of a secondary factor KLF15 (102).

Of specific interest was the observation that Cyclin D1 mRNA expression was super-induced at 18hrs after R5020 treatment in the presence of cycloheximide, suggesting that PR/R5020, may also induce the expression of proteins that regulate Cyclin D1 mRNA stability (Figure 2.5). Together these findings highlight an unexpected complexity in the mechanisms by which Cyclin D1 activity is regulated by progestins. The focus of the studies in this chapter is on the mechanisms by which PR induces the expression of Cyclin D1 mRNA. The potential importance of PR as a regulator of Cyclin D1 mRNA stability will be discussed in Chapter 3.

2.2.6 PR-B is the more robust inducer of Cyclin D1

Within most target tissues in humans PR exists is two forms, PR-A and PR-B, both of which are transcribed from different promoters within the same gene. Interestingly, although these proteins have identical DNA-binding domains and differ only by 164 amino acids at the amino terminus (PR-B being the largest protein) they have been shown to have dramatically different roles in progestin action and to have differences in target gene preferences (8). Thus, we next explored the roles of each of these two receptor isoforms in the regulation of Cyclin D1 expression. To this end, we

used a PR negative subclone of T47D (T47D:C42 cells) stably expressing Gal4 DBD (control), PR-A, or PR-B. Synchronized cells were then treated with R5020 for the time points as indicated and it was demonstrated that PR-B activation resulted in a much more robust induction of Cyclin D1 mRNA expression than PR-A (Figure 2.6). For this reason we used PR-B expressing cells for our continued analysis of Cyclin D1 expression.

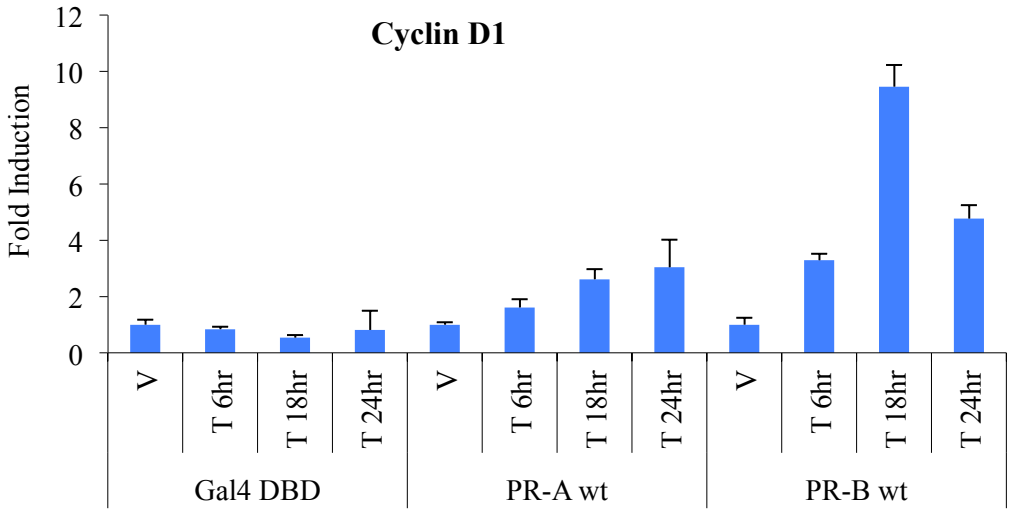


Figure 2.6: PR-B is the dominant isoform in the induction of Cyclin D1

T47D:C42 cells were transfected wih Gal4 DBD, PR-A wt or PR-B wt expression plasmids. The cells were then synchronized and treated with Vehicle (Veh) or 100pM R5020 (T) for the time points indicated. After treatment, cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3).

2.2.7 Cyclin D1 is a PR target in other breast cancer cell lines

Importantly, we also observed a 2.5 fold increase in the Cyclin D1 mRNA levels upon R5020 treatment in the PR-positive BT483 breast cancer cell line (Figure 2.7), indicating that the regulation of Cyclin D1 by PR is not a peculiarity of T47D cells.

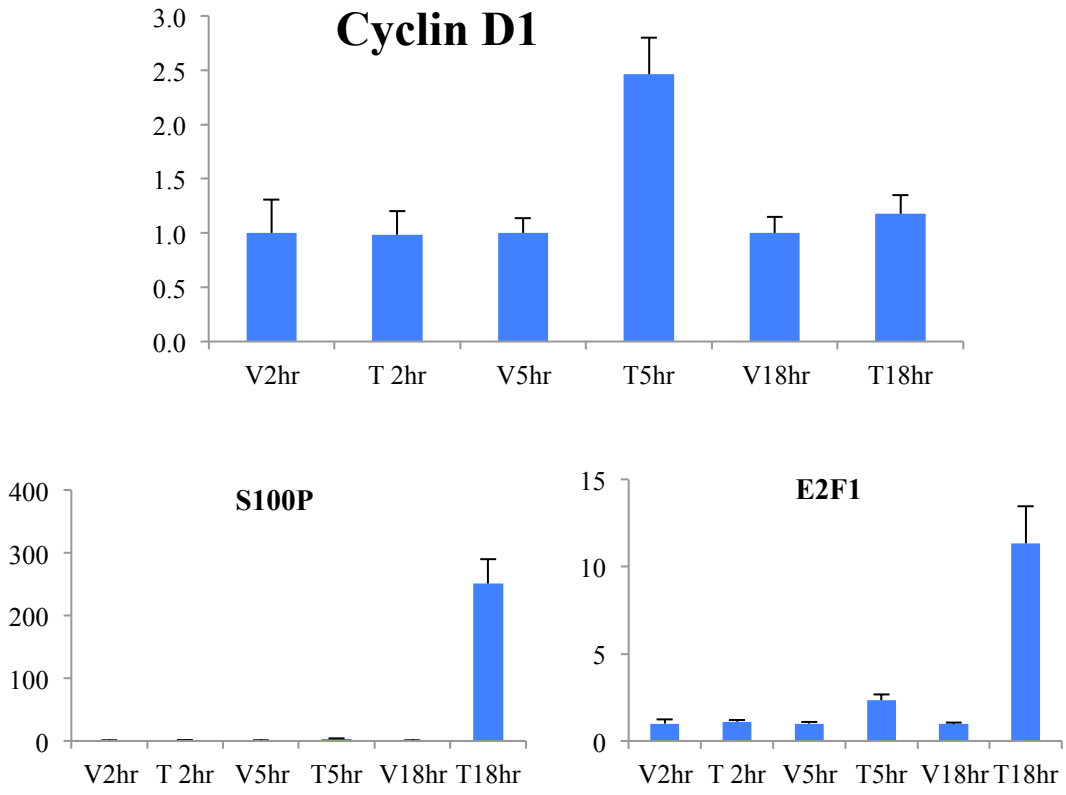


Figure 2.7: Cyclin D1 is a PR target gene in BT483 cells.

Synchronized BT483 cells were treated with Vehicle (V) or 100pM R5020 for the time-points indicated. After treatment, cells were lysed and RNA was isolated and Cyclin D1, S100P and E2F1 mRNA levels were quantified using qPCR and normalized to the expression of the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). The expression of S100P and E2F1 in R5020 treated cells is presented for comparative purposes.

2.2.8 Identification of PREs within the Cyclin D1 gene

Several groups have attempted, without success, to define specific functional progesterone response elements (PREs) within the regulatory regions of the Cyclin D1 gene. Indeed, there are several reports suggesting that although Cyclin D1 is a direct target of PR that the receptor did not interact with directly with DNA but rather was tethered by another pre-bound transcription factor. One study indicated that PR was tethered to an AP-1 complex while others proposed a tethering model involving the Erb-B2/Stat3 complex (113, 114). However, we were unable to confirm PR recruitment to the DNA elements implicated in these published studies in T47D cells using ChIP assays.

Bioinformatic analysis of the DNA sequence 50kb upstream and downstream of the transcription start site of the Cyclin D1 gene revealed several sequences that resemble canonical PREs with the palindromic sequence agaacannntgttct. However, recruitment of PR to these putative binding sites could not be demonstrated using ChIP analysis. Recently, it has been shown that PRE half-sites are the most common elements in target genes as opposed to canonical PRE palindromes (115). Studies have demonstrated that PR monomers can bind to and activate transcription from a cluster of PRE half-sites (116-120). For this reason we also used ChIP- scanning to evaluate the functionality of several very prominent half-sites within the Cyclin D1 gene but again were unable to show that these elements were able to recruit PR.

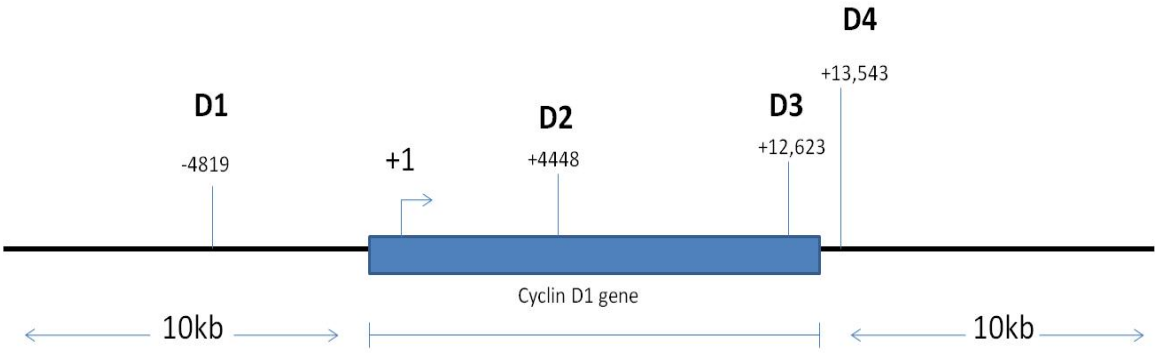
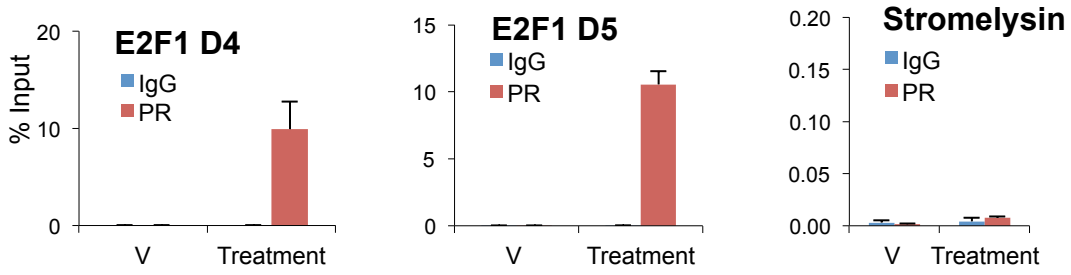


Figure 2.8: Schematic depicting the locations of regulatory elements on and proximal to the Cyclin D1 gene.

Using a bioinformatics approach to probe available ChIP-seq datasets, we determined that there were several well annotated glucocorticoid receptor (GR) binding sites within the Cyclin D1 gene (Figure 2.8). This is significant as the DNA binding site preferences of PR and GR are functionally indistinguishable (121). These GR-binding regions, ~ 250bp in length, were identified in A549 cells treated with dexamethasone and thus we next explored whether they could recruit PR in the context of T47D cells. Using ChIP analysis it was determined that PR interacted with three (denoted D2-D4) of these "GR-binding sites" in R5020 treated T47D cells (Figure 2.9).

A)



B)

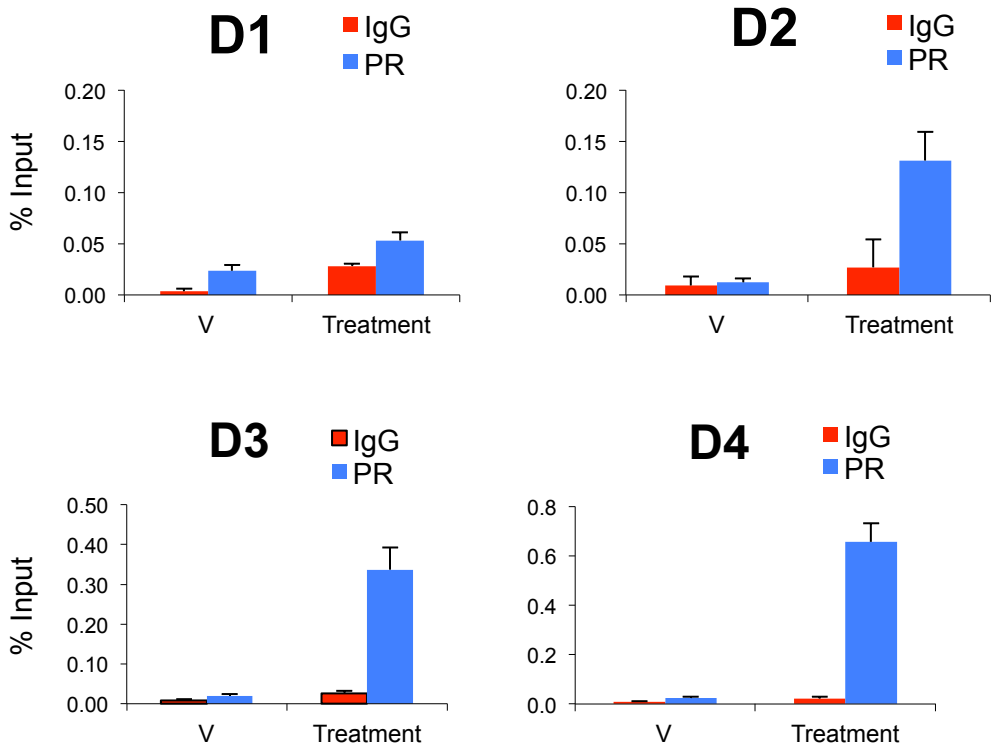


Figure 2.9: PR is recruited to regions within and proximal to the Cyclin D1 gene.

A) Synchronized T47D:A18 cells were treated with vehicle (V) or 100pM R5020 (Treatment) for 2hrs. Cells were harvested after cross-linking and subjected to immunoprecipitation with either mouse IgG control (mIgG) or PR antibody. Following reversal of cross-linking, DNA was isolated and subjected to qPCR analysis using primers spanning two regions proximal to E2F1- E2F1 D4 and E2F1 D5 (positive control), stromelysin (negative control) or B) putative PR-binding regions on and proximal to Cyclin D1 (D1-D4). The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment.

2.2.9 Assessment of the functionality of PR-binding regions identified within the Cyclin D1 gene.

The functionality of the PR-binding regions identified by ChIP (D2, D3 and D4) were next evaluated for their ability to confer progestin responsiveness upon a heterologous promoter. To this end each element was cloned into a luciferase reporter gene that contained a minimal promoter (pGL4.26 plasmid). HepG2 cells were transiently transfected with these reporter plasmids together with a PR expression plasmid and progesterone responsive luciferase expression was measured following treatment with R5020 for 24-48 hrs. The results of this study revealed that one of the elements (D2) evaluated (Figure 2.10) conferred robust PR responsiveness to the minimal promoter. Despite being able to interact with PR the D3 and D4 elements were not found to be active in this assay. Regardless we conclude from these studies that the Cyclin D1 gene contains a PR-dependent enhancer located toward the middle of the gene.

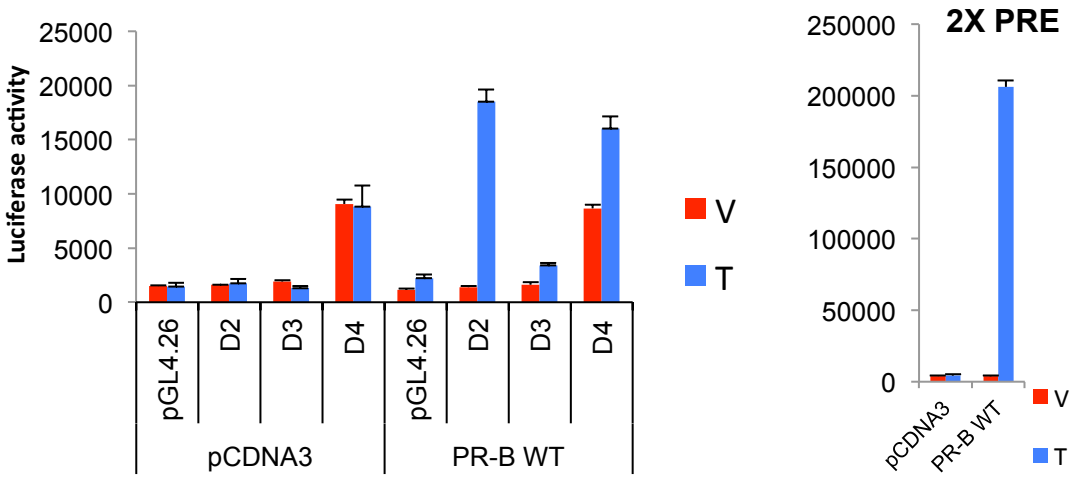


Figure 2.10: Ligand bound PR induces transcription on the D2 element.

Synchronized HepG2 cells were transfected with pGL4.26 (empty vector), or derivatives thereof containing either the D2, D3 or D4 enhancer elements cloned in front of a minimal promoter together with a vector expressing PR-B WT, its matching control vector and pCMV-β-Gal. Right-hand panel -Cells were transfected with a tk-luc vector containing a 2X PRE in front of the minimal promoter as a positive control for transcription activity. Following transfection, cells were treated for 24 hr with vehicle (V) or 100pM R5020 (T) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response obtained by normalizing luciferase activity with β-galactosidase activity.

We observed that the D4 element supported substantial basal activity independent of the PR status of the cell. This raised the possibility that the activity of another transcription factor may, in the context of this fragment, mask the activity of a PR-dependent enhancer. To address this issue we narrowed the sequence under investigation to 250bp, restricted to the putative PRE; however this fragment was also unable to support PR-mediated induction of reporter activity. We concluded from these studies that the D2 element was likely to be the primary mediator of PR-responsiveness on the Cyclin 1 gene.

Further continuing in our efforts to investigate the lack of hormone responsiveness of D3 and D4, we observed that D3 and D4 are proximal elements located towards the end and beyond the 3'UTR of the gene. Hence we wondered if they were co-dependent and might perform as enhancers when cloned in unison as one element. To this end, we constructed a pGL4.26 reporter plasmid which contained the entire genomic region from the beginning of D3 to the end of D4. However even this expanded element did not yield the results we were able to achieve with the D2 element (data not shown).

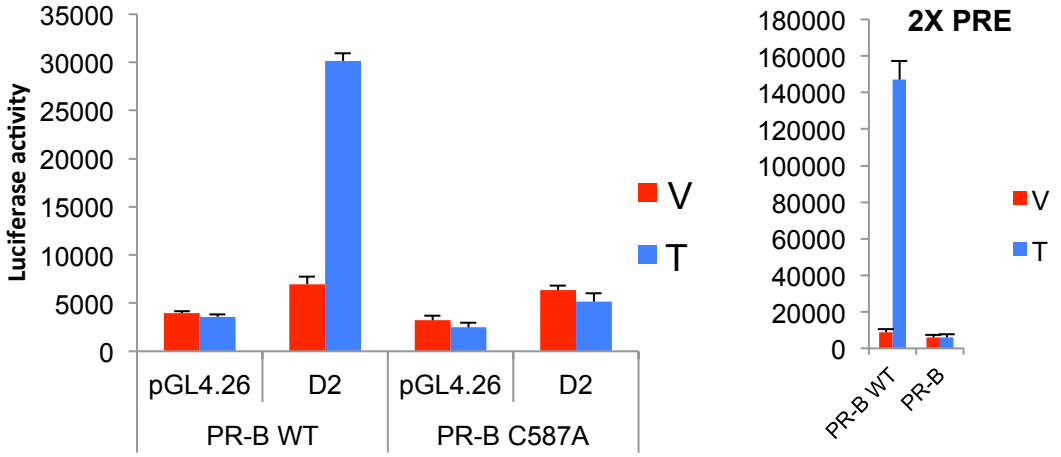


Figure 2.11: The DNA binding domain of PR is necessary for activation of D2 region.

HepG2 cells were transfected with pGL4.26 empty vector or pGL4.26 with D2, and PR-B WT or the DNA binding domain mutant PR-B C587A and pCMV-β-Gal. Right-hand panel - Cells were transfected with a tk-luc vector containing a 2X PRE in front of the minimal promoter as a positive control for transcription activity. Following transfection, cells were induced for 24 hr with Vehicle (V) or 100pM R5020 (T) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response obtained by normalizing luciferase activity with β-galactosidase activity.

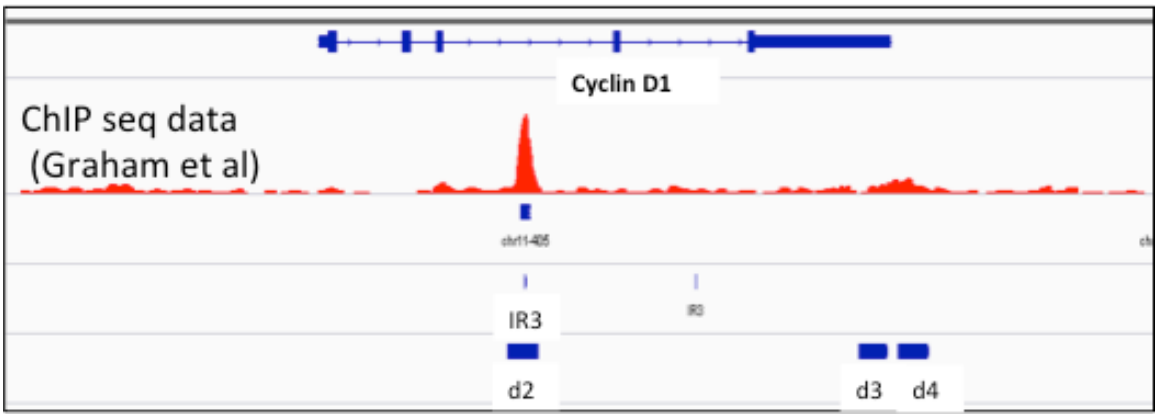
2.2.10 PR directly binds to the DNA of the response element to enhance transcription.

Whereas the D2 element conferred progestin responsiveness to a heterologous promoter it was not clear, given the lack of an obvious PRE, if this activity resulted from a direct interaction of PR with DNA or if it reflected a tethering response. To address this issue we compared the activity of WT PR and PR-B C587A, a PR expression plasmid that contains a mutation in the zinc finger domain of PR-B that renders it unable to bind to DNA, on the D2-luciferase plasmid in HepG2 cells (Figure 2.11). The results of this experiment provided definitive proof that an intact DNA binding domain was required to enable PR to interact with D2 and that a mutant capable of participating in a tethering response was inactive in this assay.

2.2.11 Identification of a 16bp specific PRE within the D2 element.

Whereas the D2 region identified was able to support PR responsiveness the nature of the specific PR interacting sequence within this fragment was not apparent. Interestingly, mining of a recently published PR ChIP-seq data set generated in ORG2058 (synthetic progestin) treated T47D cells revealed also that PR bound to the D2 region we had identified (122) (Figure 2.12 A). Using this high quality data set we performed a position weight matrix analysis and identified a 16bp sub-sequence (heretofore referred to as PRE) within D2 that exhibited the characteristics of a PRE (Fig 2.12 B). Importantly, using PCR-mediated mutagenesis, the 16bp PRE was deleted from D2 region in the reporter vector and the resultant fragment was unable to support R5020-mediated induction of the reporter in HepG2 cells (Figure 2.12 C).

A)



B)

C)

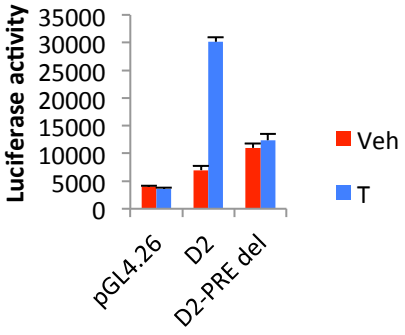


Figure 2.12: The D2 fragment of the Cyclin 1 gene contains a functional progesterone response element

A) Schematic showing the alignment of D2 element within the Cyclin D1 gene with the peak obtained by ChIP-seq by Graham et al. IR3 represents the 16bp putative progesterone response element obtained using bioinformatic techniques to calculate a position weight matrix for progesterone response elements (B) from the raw data made available by Graham et al. C) Synchronized HepG2 cells were transfected with pGL4.26 empty vector, or pGL4,26 vector with the D2 element cloned in front of the minimal promoter or with D2 with the PRE deleted (D2 PRE del) cloned in front of the minimal promoter, and PR-B WT and pCMV-β-Gal. Following transfection, cells were induced for 24-48 hr with Vehicle (V) or 100pM R5020 (T) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response, which was obtained by normalizing luciferase activity with β-galactosidase activity.

2.2.12 The 16bp PRE within the D2 region of the Cyclin D1 gene is sufficient to confer progestin responsiveness to a heterologous promoter.

While the above reporter assays were carried out in HepG2 cells due to their established strong transcriptional response to steroids, our attempts to show that D2 was a PR responsive element with the endogenous PR in T47D cells were unsuccessful (see discussion section for explanation). However, when we cloned the 16bp sequence isolated from the above bioinformatics and deletion analysis, in front of the minimal promoter in the above pGL4.26 system (PRE in Figure 2.13) and introduced the plasmid into T47D cells, we found that this element conferred modest progestin responsiveness to the luciferase reporter. In fact, transfecting in exogenous PR plasmid (PR-B or VP16 PR-B) significantly increased the PR-mediated transcription of the reporter as compared to the control or non-PR transfected cells. Briefly, VP16 PR-B is a plasmid bearing a fusion of the VP16 transactivation factor and the PR-B plasmid. Thus this plasmid gives us the ability to detect if PR-B is at least able to interact with the target DNA if not recruit

transcription factors, since the fusion plasmid can activate transcription via the VP16 factor once bound to the DNA.

A detailed description of the hypotheses to explain the above results is presented in the discussion section below. Overall, the fact that the 16bp element could act in isolation to induce transcription of the reporter from a minimal promoter led us to infer that the above identified PRE was not only necessary but also sufficient to act as an enhancer for PR-mediated transcription.

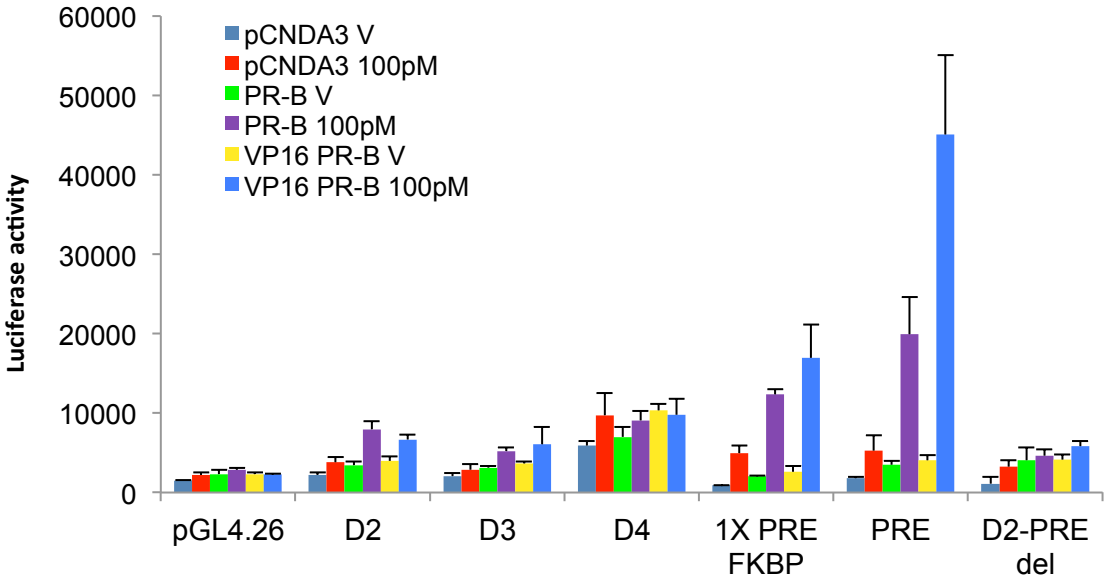


Figure 2.13: The 16bp PRE is sufficient to induce PR-mediated transcription in T47D cells.

Synchronized T47D cells were transfected with the pGL4.26 empty vector, or pGL4.26 vector with the D2, D3 or D4 enhancer element cloned in front of the minimal promoter or pGL4.26 containing a 1X PRE isolated from the promoter of the known PR primary target gene FKBP (1X PRE FKBP, positive control), or the 16bp PRE (PRE), or D2 bearing a deletion of the 16bp PRE (D2-PRE del) in front of the minimal promoter and pcDNA3 or PR-B or VP16 PR-B and pCMV-β-Gal. Following transfection, cells were induced for 24-48 hr with Vehicle (V) or 100pM R5020 (100pM) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response, which was obtained by normalizing luciferase activity with β-galactosidase activity.

2.2.13 Cyclin D1 and other growth factors

While progestins have been shown to induce Cyclin D1 in breast cancer, other growth factors such as EGF, heregulin and estrogens have also been shown to perform the same function (123-125). Thus we wondered if these growth factors could also elicit an increase in Cyclin D1 levels in T47D cells, and if the pattern of regulation was the same as that by R5020. As shown in (Figure 2.14 A), we treated T47D cells with all these growth factors for 2, 6 and 18hrs. While estradiol did not show any evident effect on Cyclin D1 across all these time-points, both EGF and Heregulin showed an upregulation in Cyclin D1 mRNA levels. EGF, along with R5020, was able to rapidly induce Cyclin D1 within 2hrs of progestin treatment. R5020 however, was the most potent inducer of Cyclin D1 at each of the time-points. It was also interesting to note that both EGF and Heregulin were able to regulate Cyclin D1 in a manner similar to R5020, with a decrease in levels seen at 18hrs as compared to 6hrs. Further experiments are required to discern whether this is just decay of an induced transcript over time, or if the mechanism of this regulation is the same as that which R5020 exerts on Cyclin D1 for induction followed by reduction in levels.

In order to determine if the actions of EGF on Cyclin D1 are delayed as compared to R5020, we carried out a second experiment wherein T47D cells were exposed to R5020 or EGF from 6hrs – 48hrs. However we were disappointed to note that the changes in Cyclin D1 levels upon EGF treatment were not as dramatic as upon R5020 treatment; while there was an induction seen at 6hrs as in (Figure 2.14 B), the levels dropped slightly and reached a plateau after 18hrs. From these experiments we conclude that while other growth factors can also induce Cyclin D1, progestins have the most compelling effect on the expression of Cyclin D1 in these cells, among the growth factors tested. It is also noteworthy that Cyclin D1 levels do not come back up once attenuated, implying that the cells cannot re-enter the cell-cycle.

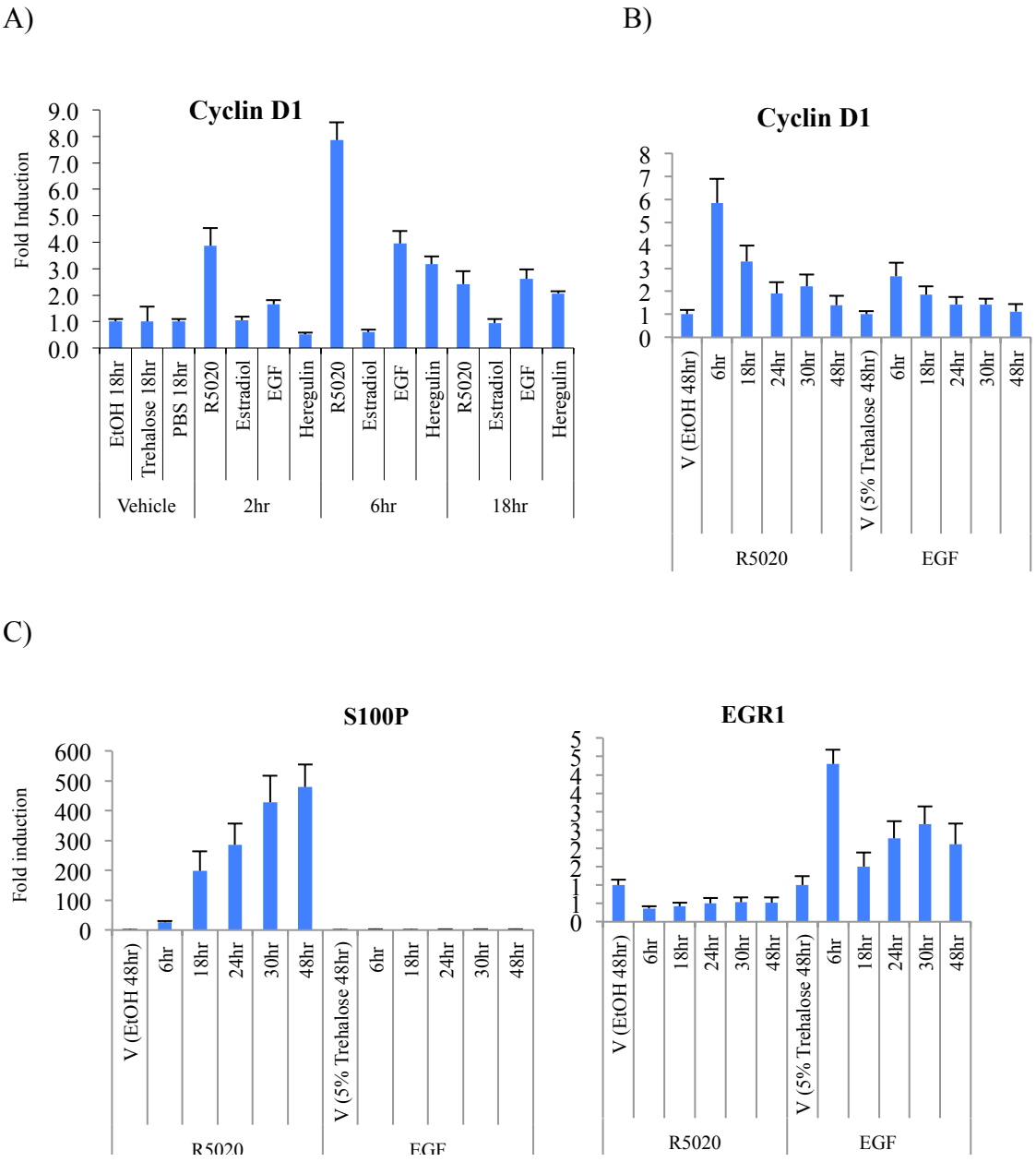


Figure 2.14: Cyclin D1 is induced by other growth factors.

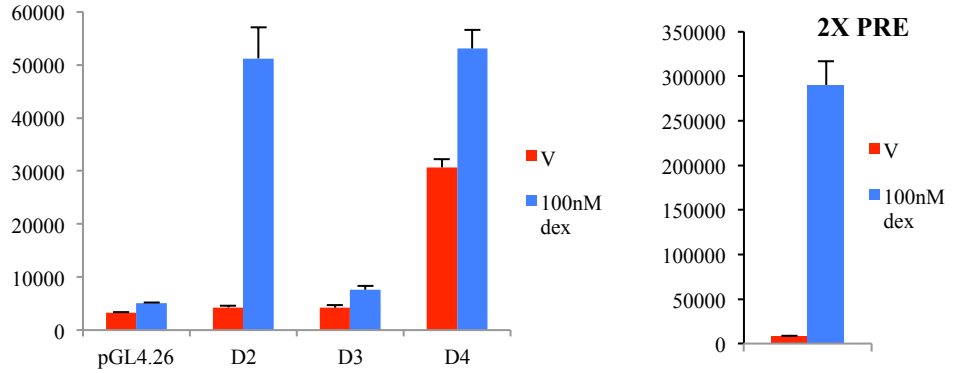
Synchronized T47D:A18 cells were treated with (A) control (EtOH, Trehalose or PBS) or treatment (R5020, Estradiol. EGF or Heregulin) for the time points indicated or (B) control (EtOH or Trehalose) or treatment (R5020 or EGF) for the time points indicated. After treatment, cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1, S100P (C) and EGR1 (C) mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). (C) S100P and EGR1 are presented as positive controls for R5020 and EGF treatment respectively.

2.2.14 GR and Cyclin D1 in breast cancer

Since the PR binding regions on Cyclin D1 were originally discovered as GR binding elements, we wondered if the GR agonist dexamethasone could compete with R5020 to occupy Cyclin D1 sites in our cells. The reporter assay confirmed that GR can bind and activate D2 (Figure 2.15 A). Upon treating T47D cells with dexamethasone for 6hrs, we observed that GR could, albeit weakly in comparison to R5020, activate Cyclin D1 levels (Figure 2.15 B). However, upon co-treatment with R5020 and dexamethasone, Cyclin D1 was induced to the same levels as with R5020 alone, which indicated that GR was not the dominating factor; the two nuclear receptors were co-occupying the response sites or R5020 was the primary transcription factor binding to the enhancers and promoter region in these cells.

However, the fact that other nuclear receptors can also bind and activate Cyclin D1 in breast cancer is significant from a therapeutic point of view. Whether GR also regulates Cyclin D1 through the same mechanisms as PR, remains to be investigated.

A)



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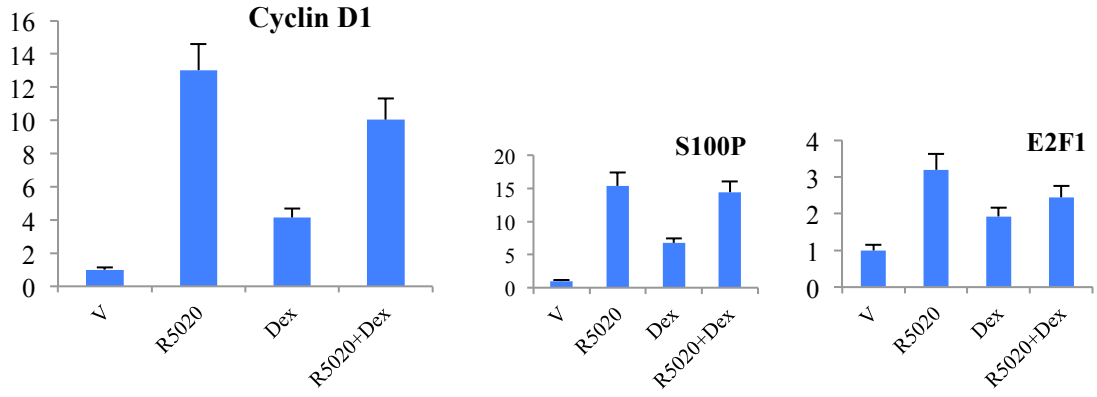


Figure 2.15: Cyclin D1 is induced by ligand-bound GR

(A) Synchronized HepG2 cells were transfected with pGL4.26 empty vector, or pGL4.26 vector with the D2, D3 or D4 enhancer element cloned in front of the minimal promoter and pcDNA3 or PR-B and pCMV-β-Gal. Right-hand panel -Cells were transfected with a tk-luc vector containing a 2X PRE in front of the minimal promoter as a positive control for transcription activity. Following transfection, cells were induced for 24 hr with Vehicle (V) or 100nM Dex (100nM Dex) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response obtained by normalizing luciferase activity with β-galactosidase activity. (B) Synchronized T47D:A18 cells were treated with Vehicle(V) or 100pM R5020 (R5020) or 100nM dexamethasone (Dex) or R5020 and dexamethoasone (R5020+Dex) for the time-points indicated. After treatment, cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1, S100P and E2F1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3).

2.3 Discussion

In this study we chose to focus on dissecting the mechanisms by which progestins regulate the phosphorylation of the tumor suppressor Rb. Using a genetic and a pharmacological approach we determined that the activation of MAPK, attributed to non-genomic activities of PR, was not responsible for Rb hyperphosphorylation as had previously been suggested. We were instead able to demonstrate that Cyclin D1 is a primary target of PR and were able to identify a specific PR-dependent enhancer within the Cyclin D1 gene. Specifically, it was shown that progestins can rapidly induce Cyclin D1 mRNA expression within 2hrs and this induction decreases by 18hrs of treatment. Guided by previously published GR ChIP seq data we used ChIP assays, to identify putative PR binding sites within and proximal to the Cyclin D1 gene. We have shown that one of those elements, D2, is a PR-responsive element that includes a specific 16bp

element that is both necessary and sufficient for PR induced transcription activity. It was concluded therefore that Cyclin D1 is a primary PR target gene and that the regulatory activities of this receptor require its direct interaction with a *bona fide* PRE within this gene.

2.3.1 Cyclin D1 and breast cancer

Given that Cyclin D1 plays such a pivotal role in proliferation, it is plausible that deregulation of this gene can lead to disarrayed cell cycle gene expression and hence tumor formation. In fact there are several lines of evidence that implicate Cyclin D1 in tumorigenesis. Mouse mammary tumor virus (MMTV)-Cyclin D1 mice have been shown to develop tumors with long latency (126). While clinical studies have shown that this gene is over-expressed in over 50% of breast cancers (127, 128), its expression is inversely related to overall and relapse-free survival in breast cancer patients (129, 130).

2.3.2 Non-canonical roles of Cyclin D1

Aside from its traditional and well-characterized role in G1-S phase transition in the cell cycle, new roles are emerging for Cyclin D1 in chromosomal instability and DNA damage repair (131), which could be significant in PR positive breast cancer pathology. For example, Casimiro et al used genome-wide ChIP sequencing and found that a DNA-bound form of Cyclin D1 occupied the regulatory region of several genes that are involved in regulating chromosomal stability. Furthermore, transgenic mice with acute and continuous mammary gland–targeted Cyclin D1 expression presented with increased tumor prevalence and exhibited a signature that overlapped with those

described previously for chromosome instability (132). Following up on the observation that Cyclin D1 is colocalized to dsDNA break spots along with Rad51, and the fact that cells depleted of Cyclin D1 showed reduced Rad51 recruitment to sites of DNA damage and reduced rate of homologous recombination based DNA repair (133), Jirawatnotai and co-workers suggested a role for Cyclin D1 in mediating the repair of ds breaks in DNA. Studies performed in mammary epithelial cells, as well as *Cyclin D1* -/- bone marrow macrophages, have also identified a significant role for Cyclin D1 in cell adhesion and migration (134, 135). The above results warrant further investigation of the non-canonical functions of the induced Cyclin D1 in PR positive breast cancer progression.

2.3.3 Reporter activity in T47D cells

In spite of repeated efforts with higher doses, we were unable to obtain the desired induction of reporter activity with the pGL4.26-D2 plasmid in T47D cells. However, we observed an increase in luciferase activity upon co-transfection with PR-B or the VP16 PR-B plasmid, with the latter giving a higher magnitude of induction. VP16 is a 65 kD activating factor that primes transcription from the five virally encoded immediate early (IE) genes (136). VP16 PR-B is a fusion of this factor and PR-B that will thus increase the efficiency of transcriptional activation as long as the fusion protein is bound to DNA via the PR-B domain.

While this is counter-intuitive given that T47D:A18 cells already express high levels of endogenous PR-B, this anomaly has been encountered in our group with other nuclear receptors such as ER and AR. We suspect that endogenous ligand-bound PR may

be unable to (i) bind to this specific reporter construct or (ii) recruit transcription machinery to the promoter due to the presence of unknown suppressor elements on D2 itself that are inhibiting transcription initation. In fact, the observation that the VP16 PR-B plasmid, led to greater activation of the reporter as compared to PR-B alone prompted us to hypothesize that the problem encountered by endogenous PR-B in T47D cells was not the binding of DNA but the recruitment of the transcription machinery to the active site. The validity of these hypotheses is yet to be confirmed by our group.

2.3.4 Elements D3 and D4 in Cyclin D1 activation

While ChIP studies demonstrated that these regions had a greater degree of PR recruitment, PR had little to no activity on the D3 and D4 regions identified in the bioinformatics analysis. Thus we concluded that at least within the context of the model systems we are using they are unlikely to serve as PREs. Possible reasons include the fact that other genomic elements not present in the construct might be needed for these regions to be functional. A repressor element might be hidden within the fragment that is blocked in the endogenous cell, or these regions might simply serve as tethering stations for PR to bring in other factors to activate transcription at an active region such as D2.

Interestingly, in our attempts to probe further into the lack of progestin response of the other PR-binding elements uncovered that other well-known nuclear receptor co-factors such as FoxA1 and GATA3 are bound to D3 and D4 under just basal conditions. (137). In fact, DNase studies under DMSO treatment conditions showed an enrichment of peaks in that region, implying that this is an open chromatin region when the cell is not

subject to any extracellular ligands (Figure 2.16). Additionally, studies on MCF7 cells treated with estradiol show that ER is also bound to those regions, along with FoxA1 and GATA3 (data not shown). Thus, one might speculate that these regions are open binding regions even in a resting state due to being bound by pioneering factors like FoxA1 and GATA3. They are thus promiscuous in terms of their binding proteins, which explains the recruitment of R5020 activated PR to these regions in our ChIP assays. These open regions might also be required to regulate the chromatin architecture at enhancer sites along and proximal to the Cyclin D1 gene. Further studies are certainly warranted in order to test these hypotheses.

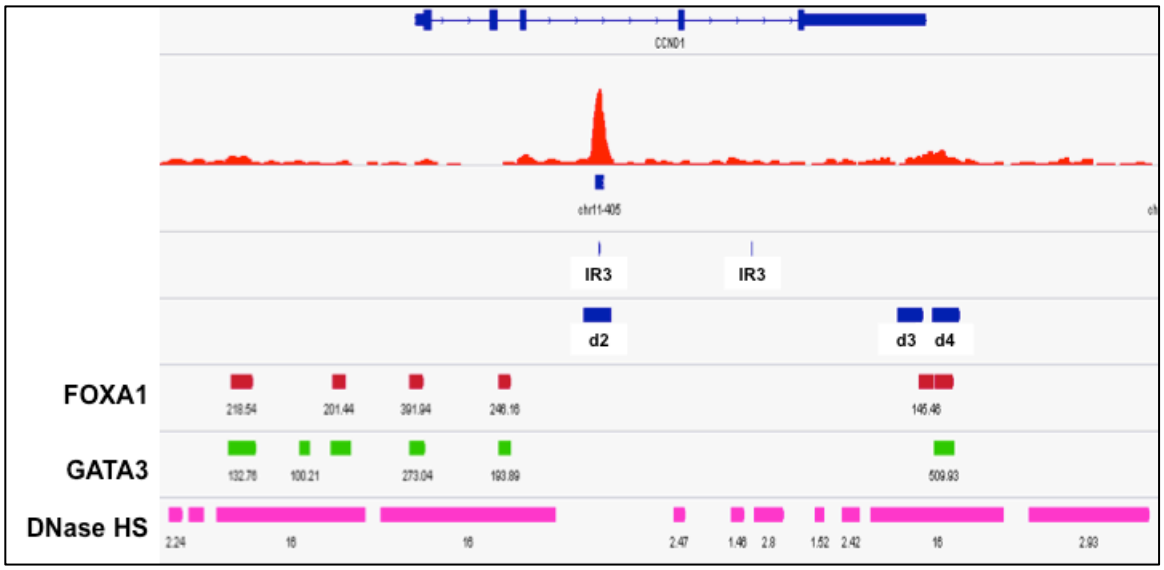


Figure 2.16: Regions D3 and D4 are open chromatin regions that bind other pioneering factors under basal conditions.

IR3 indicates the PRE identified by the PR position weight matrix identified by bioinformatics analysis of the ChIP seq data in Graham et al. PR binding regions D2-D4 (indicated by SK PR ChIP sites) are aligned to indicate their position along the Cyclin D1 genomic region. Following that are binding regions for FoxA1, GATA3 and DNase hypersensitivity sites, aligned along and surrounding the Cyclin D1 gene.

In conclusion, our studies provide evidence for the inference that PR induction of Rb phosphorylation is achieved by the induction of the Cyclin D1 gene by direct binding to a PR-responsive Cyclin D1 genomic region. We believe that these studies are significant in understanding the debated phenomenon of PR regulation of Cyclin D1, a gene that is important from both a cell cycle and tumorigenesis perspective as well as it's roles in other non-canonical phenotypes in breast cancer cells.

3 Regulation of Cyclin D1 mRNA by progestins

3.1 Introduction

The progesterone receptor belongs to the family of nuclear receptor transcription factors that are sequestered in the cytoplasm when inactive and in the absence of ligand. Upon exposure to agonist, the receptor undergoes a conformational change, dimerizes and translocates to the nucleus, where it can interact with available co-factors and enable the transcription of target genes.

3.1.1 The use of anti-progestins in breast cancer

A majority of breast cancers are hormone receptor positive and ER is expressed in 75% of these cancers, and within the ER positive breast cancers, more than half of these also express PR (79). The Women's Health Initiative trial in 2002 brought the first piece of evidence suggesting that progestins may have a role in breast cancer tumorigenesis (138). The trial was undertaken to evaluate the effects of combined hormone replacement therapy (HRT) of progestins and estrogens Vs estrogen treatment alone. However, the trial was suspended pre-maturely because the results showed that women receiving both progestin and estrogen were at greater risk for breast cancer than women receiving estrogen alone. The Scandinavian HABITS (Hormone replacement therapy After Breast cancer- Is It Safe?) trial was also terminated early because of similar findings that women receiving combined HRT were at greater risk of recurrence (82). In fact, oral contraceptives given as combinations of both hormones have also been implicated in breast cancer risk (139). Extensive analysis of these data suggests that progestins may

contribute to breast cancer progression, indicating that antiprogestins like mifepristone (RU486) could be a useful anti-hormonal intervention. Initial studies of RU486 in animal models showed promising results, namely that RU486 was as effective as the antiestrogen tamoxifen at inhibiting breast tumor growth (84). However, in a series of small clinical trials, RU486 was shown to have only a moderate response rate in patients with metastatic, tamoxifen-resistant cancer (85). A major limitation of these studies, however, was that patients were enrolled independent of PR and ER status. A similar shortcoming in the trial of the pure antiprogestin onapristone (ZK 98 299), resulted in positive although not statistically significant benefit (86).

3.1.2 The role of PR in the breast cancer cell cycle

On a whole, the above mentioned results indicate that progesterone is playing a significant role in breast cancer and a better understanding of how PR influences cellular growth in breast cancer is needed. Progestins have been shown to modulate cellular proliferation *in vitro* as well as in animal models of breast cancer (94). In PR+/ER+ T47D cells, synthetic progestin treatment results in an acceleration of cells through one passage of the cell cycle followed by arrest in early G1 (95). The rapid induction of Cyclin D1 and the proto-oncogene cMyc by progestins have been cited as possible candidates responsible for the initial G1-S phase transition. The sequence of events following Cyclin D1 expression was shown to parallel that of the response upon mitogen stimulation, namely the activation of Cdk2 and phosphorylation of the tumor suppressor Rb protein. This phosphorylation event leads to the release of E2F, which induces the

transcription of cell-cycle genes to enable G1-S transition (95-97). In contrast, the subsequent cell cycle arrest correlates with a decrease in Cyclins D and E and downregulation and dephosphorylation of Rb, as well as induction of Cyclin-dependent kinase (CDK) inhibitors p21 and p27(Kip1) and the association of Cyclin D-cdk4 and Cyclin E-cdk2 complexes with these inhibitors (98, 99). On the other hand, treatment with anti-progestins inhibits the growth of T47D cells. Increased p21 expression and synchronous inhibition of CDK activity have been suggested as mechanisms for the above phenomenon (100). Interestingly, the CDK activity inhibition was significantly reduced upon ectopic expression of Cyclin D1, suggesting that Cyclin D1 is an important target of the cell cycle machinery that plays a critical role in anti-progestin mediated growth inhibition. Thus, while several molecular targets of progestin treatment in breast cancer cells have been elucidated, the overall mechanisms governing the proliferative and anti-proliferative mechanisms of progestins remain unclear.

In agreement with other labs, we have also shown a rapid induction of Cyclin D1 by progestins in breast cancer cells. Interestingly, it was determined that progestin mediated up-regulation of Cyclin D1 is rapid, peaking at 6hrs post hormone addition followed by a decrease in expression at 18hrs. This pattern of regulation is synchronous with the characteristic passage of these cells through S phase starting around 8-10hrs after progestin treatment, and then through one round of the cell cycle by 22hrs followed by arrest in G1 (95). Unexpectedly, we found that the induction of Cyclin D1 mRNA expression was a primary transcriptional event and have demonstrated the specific interaction of PR with a PRE located in the middle of the gene. However, the mechanism

of Cyclin D1 mRNA levels declining by 18hrs had yet to be determined. We postulated that delineating the pathway(s) that lead to this decline in mRNA levels at the later time point could lead to an explanation of the characteristic cell-cycle response of breast cancer *in vitro* models to progestins, and thus provide potential insights into the factors that drive PR-mediated cellular proliferation.

3.2 Results

3.2.1 Cyclin D1 half-life decreases over time upon treatment with progestins

Upon treatment of T47D:A18 breast cancer cells with progestins, we found a rapid increase of Cyclin D1 mRNA and protein levels within 2hrs, followed by a decrease in expression by 18hrs. In order to determine if Cyclin D1 was a direct target of progestins, we pre-treated our cells with cycloheximide followed by R5020. While the initial induction of mRNA levels remained unaffected, implying that Cyclin D1 was a primary PR target, we observed that the mRNA levels at 18hrs were super-induced compared to 6hrs (Figure 2.5, Chapter 2). Based on this result, we hypothesized that a secondary mRNA destabilizing factor was suppressing the 18hr transcript. To test this hypothesis, we used Actinomycin D, which interferes with mRNA synthesis and halts transcription, to assess if the half-life of the 18hr transcript was less than the 6hr transcript. Synchronized T47D cells were treated with R5020 for 6 or 18hr, at which point the cells were exposed to Actinomycin D to halt transcription (Figure 3.1). The RNA was then collected every hour for up to 4 hours and Cyclin D1 expression was quantified by qPCR to evaluate the rate at which the RNA levels were declining in the

event of no active transcription. The presence of a secondary mRNA destabilizing factor at 18hr would lead to increased mRNA degradation, and therefore a shorter half-life. We were excited to discover that the half-life of the 18hr transcript was indeed lower than that of the 6hr transcript. Based on these results, we concluded that one of the mechanisms by in which progestins attenuate the levels of Cyclin D1 is through the regulation of it's mRNA stability. We hypothesized that PR induces an mRNA binding protein which causes destabilization of Cyclin D1 mRNA.

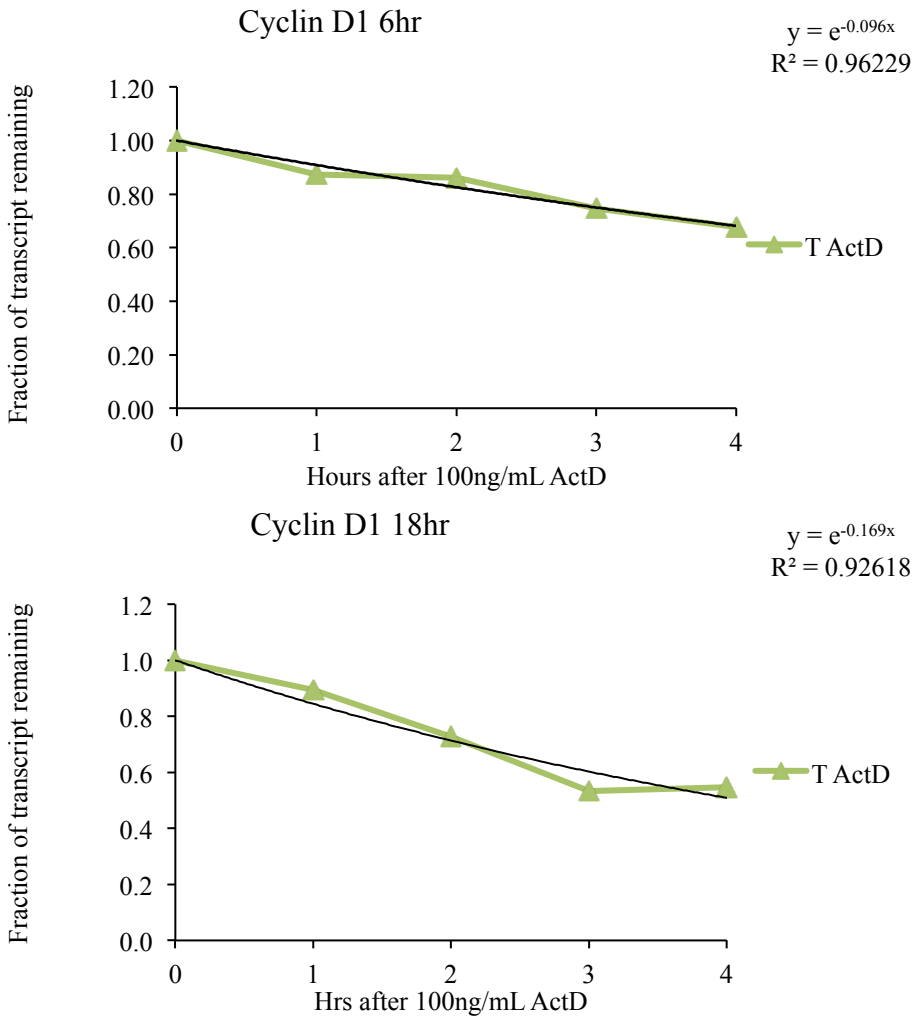


Figure 3.1: Progestins decrease the stability of the Cyclin D1 mRNA over time.

Synchronized T47D:A18 cells were treated with 100pM R5020 (T) for the time points indicated (6hr or 18hr). After treatment for the respective time, cells were treated with the transcription inhibitor Actinomyosin D. RNA was then collected after the time points indicated (1,2,3, or 4hr). Cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. The RNA levels of the non-actinomycin treated control cells was set to 1 and RNA collected at each time is represented as a fraction of the control RNA. Results are expressed as fold induction over vehicle-treated cells at 6 or 18hr ± SEM (n=3).

3.2.2 The RNA binding protein tristetraprolin is induced by progestins

Based on our previous analysis, we chose to investigate RNA binding proteins as potential candidates for the progestin-induced secondary factor that lowers Cyclin D1 half-life. We chose to focus on the ARE-BP (AU-rich element binding protein) family, which are a family of proteins that bind to the AU-rich elements of the 3' UTR regions of target mRNAs and thus modulate their stability.

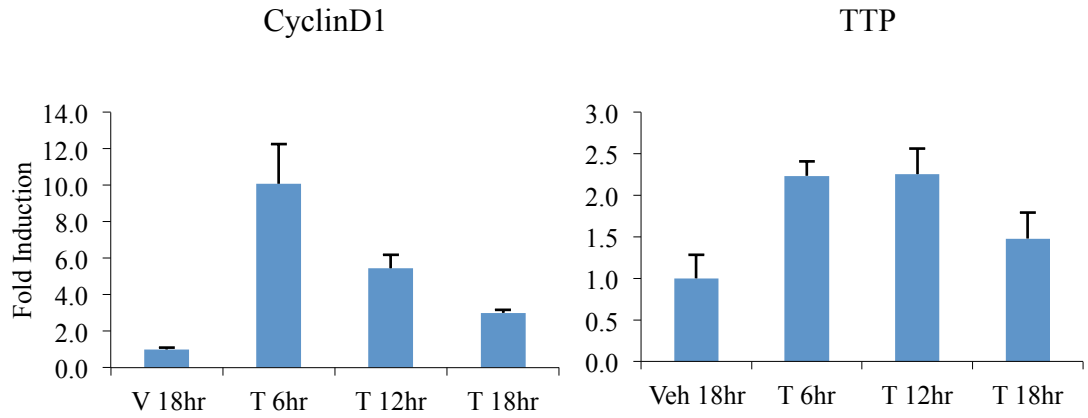
We initially performed qPCR analysis of a subset of ARE-BP family protein members that we identified based on microarray data analysis and a literature search. To establish the candidate list, we first looked at previously generated microarray data. A microarray study performed by Mrusek et al in 2005 on T47D cells treated with estradiol and medroxyhydroprogesterone (MPA) showed a 2.8-3.5 fold increase in tristetraprolin (TTP) mRNA expression levels, a well known RNA binding protein that targets the post-transcriptional modification of cytokines (140). Upon examination of previously performed T47D microarray datasets within our lab for candidate RNA binding proteins, we found a dataset that showed an induction of TTP with progestins. A microarray analysis performed by a previous lab member Sakiko Kobayashi in non-transformed, normal human mammary epithelial cells (hMECs) (where hPR-B is transiently expressed) in the presence and absence of R5020 for 16hrs, showed that TTP was 2.9 fold induced. Two other ARE-BP family members, BRF1 and BRF2, were shown to be R5020 repressed in the latter microarray. Since these are mRNA destabilizing factors (141) that are repressed by PR, we did not consider them as likely candidates for our

hypothesis. Based on the above microarray analysis, we chose to focus on TTP as one of the likely candidates.

In addition to the microarray data supporting PR-mediated TTP induction, TTP has also been shown to modify Cyclin D1 mRNA stability upon exposure to rapamycin via p38 MAPK signaling (142). Moreover, a literature search enabled us to identify additional ARE-BPs that had been shown to associate with Cyclin D1 and negatively or positively impact its mRNA stability (143-145) - AUF1 and HuR and SNIP1.

Thus, based on the above data, we proceeded to evaluate TTP, AUF1, HuR and SNIP1 expression levels via qPCR analysis in synchronized T47D cells stimulated with R5020 for various time points. Within this analysis, TTP was the only candidate that was up-regulated and it followed a similar pattern of time dependent regulation as Cyclin D1 itself (Figure 3.2 A). We were also able to see this enrichment at the protein level (Figure 3.2 B).

A)



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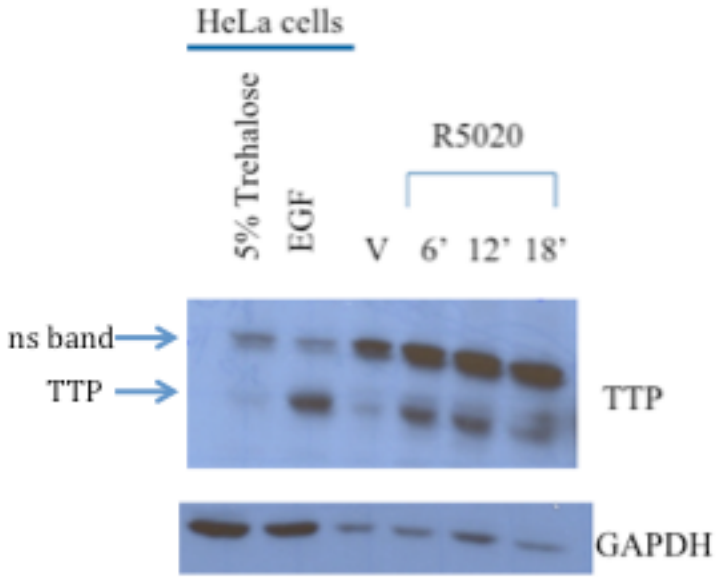


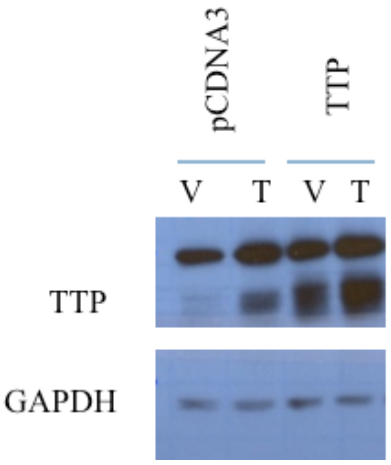
Figure 3.2: TTP is induced by progestins in breast cancer cells.

Synchronized T47D:A18 cells were treated with Vehicle (V) or 100pM R5020 (T) for the time-points indicated. A) After treatment, cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1 and TTP mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). B) After treatment, whole cell extract was subjected to immunoblotting for TTP and GAPDH (loading control). TTP induced by EGF in HeLa cells in the two left-most lanes is presented as a positive control to confirm the right band for TTP since the antibody also gives rise to a non-specific (ns) band.

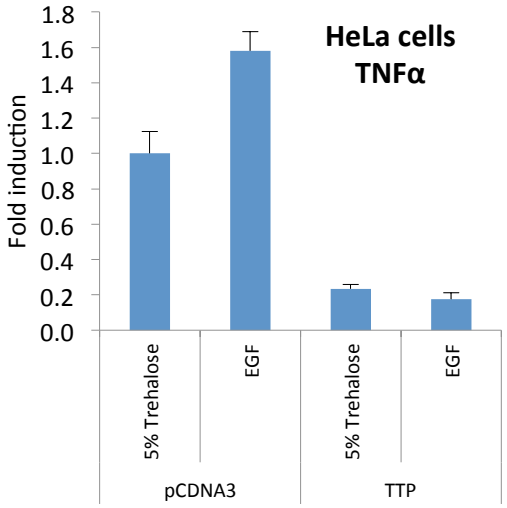
3.2.3 Evaluation of the effect of TTP overexpression on Cyclin D1 mRNA stability

In order to investigate the role of TTP in Cyclin D1 mRNA stability, we obtained a TTP expression plasmid from a collaborating lab (Keene Lab, Duke University) that we transiently transfected into T47D cells. Figure 3.3A shows we were able to achieve overexpression of the TTP protein beyond the R5020 mediated induction. In order to demonstrate that this overexpression is functional, we transiently transfected HeLa cells which show enrichment of TTP under EGF stimulation, with the TTP expression construct. Figure 3.3B shows that the TTP overexpressing cells have significantly lower levels of the well-established target gene TNFα even under non-EGF treated conditions. However, when we assessed the Cyclin D1 mRNA levels in cells overexpressing TTP at 6 or 18hr after treatment, we were unable to find an effect on Cyclin D1 levels at 18hrs (Figure 3.3C). Unfortunately despite several attempts, we were unable to obtain two reliable TTP siRNA sequences that did not exhibit off-target effects. However on the basis of the over-expression experiment, we concluded that Cyclin D1 is not one of the target genes of PR-induced TTP.

A)



C)



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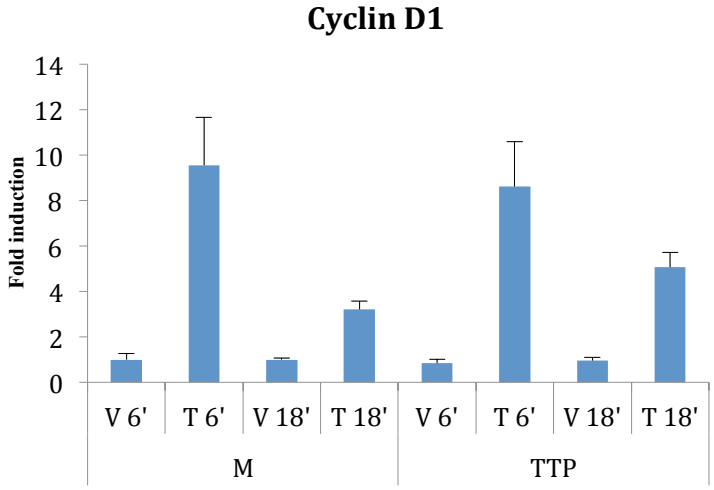


Figure 3.3: TTP overexpression in breast cancer cells.

A and B) T47D cells were transfected with pcDNA3 or TTP expression plasmids and then serum starved for 24hrs. A) Cells were then treated with V or 100pM R5020 for 18hrs. After treatment, whole cell extract was subjected to immunoblotting for TTP and GAPDH (loading control). B) Cells were treated with V or 100pM R5020 for 6 or 18hrs. After treatment cells were lysed and RNA was isolated and reverse transcribed. Cyclin D1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. C) HeLa cells were transfected with pcDNA3 or TTP expression plasmids followed by treatment with 5% Trehalose or EGF for two hrs. After treatment cells were lysed and RNA was isolated and reverse transcribed. TNFα mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. qPCR results are expressed as fold induction over vehicle-treated cells ± SEM (n=3).
TNFα is presented as a positive control for functional overexpression of TTP in HeLa cells.

3.2.4 TTP represses inflammatory genes in breast cancer cells

Although we were unable to establish Cyclin D1 as a target of TTP action, we wished to explore the function of progestin-induced TTP in breast cancer. Previous groups have demonstrated that TTP diminishes the lipopolysaccharide (LPS)-induced expression of several pro-inflammatory genes such as TNFα, IL6, Cox2, CCL2 etc (146, 147) by negatively regulating their stability. We found that treatment of T47D cells with the cytokine interleukin-1β (IL-1β), lead to activation of several NF-κB and other inflammatory genes, while non-inflammatory genes such as HIF-1α were not modified, indicating this is a specific effect. Thus we wondered if overexpression of TTP under IL-1β stimulated conditions would impact the activation of these genes. As predicted, Figure 3.4 shows that TTP was indeed able to attenuate the up-regulation of these genes upon IL-1β exposure.

Interestingly, a previous study in our lab led by Sokiko Kobayashi (148) demonstrated that agonist-bound PR was able to suppress the NF-κB activation of several

target genes such as CCL2 and CCL20. We were therefore very interested in determining if the two phenomena were convergent and more specifically, was TTP a mediator in the inhibition of NF-κB target gene suppression by progestins. However, we were unable to validate this hypothesis in our cell lines due to the absence of reliable siRNA tools. While TTP inhibitors are not yet commercially available, we propose to establish stable T47D cell lines harboring shRNA-mediated TTP knockdown and thus continue the investigation of this hypothesis in our group.

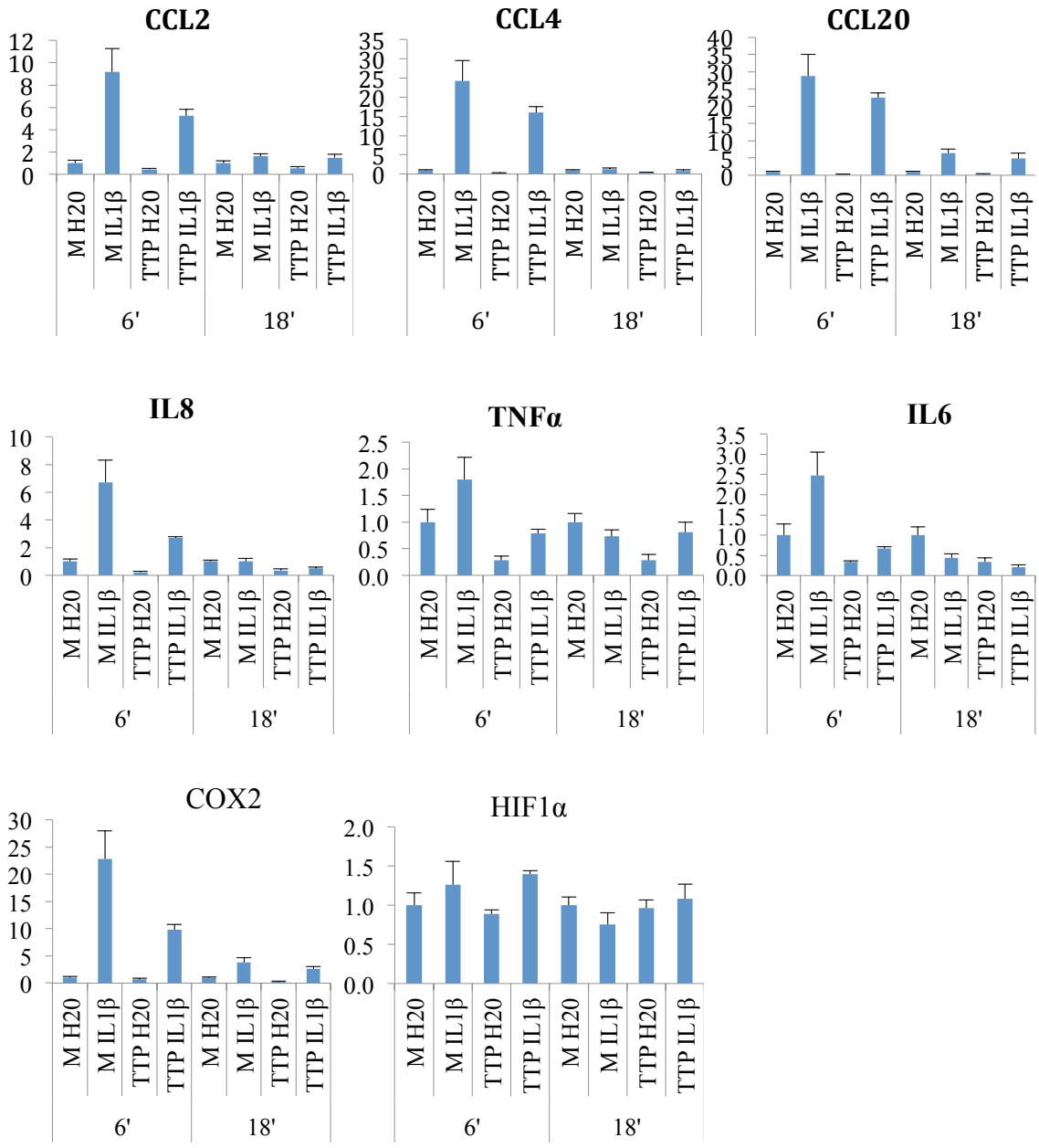


Figure 3.4: TTP represses inflammatory genes in breast cancer cells

T47D:A18 cells were transfected with mock (M), i.e pcDNA3 or TTP expression plasmid, followed by treatment with IL-1β for 6 or 18hr. After treatment cells were lysed and RNA was isolated and reverse transcribed. mRNA levels of the various genes were quantified using qPCR and normalized to the housekeeping gene 36B4. qPCR results are expressed as fold induction over vehicle-treated cells ± SEM (n=3).

3.2.5 PAR-CLIP databases for the identification of known Cyclin D1 3'UTR binding proteins.

Since our initial literature screen did not identify the secondary factor that enabled mRNA half-life decline at 18hrs, we expanded our search to proteins that had been identified via PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation). PAR-CLIP is a technique that relies on the incorporation of photoreactive ribonucleoside analogs into nascent RNAs in cells, to identify sites of binding of RNA binding proteins. Through a collaboration with another graduate student (Matthew Friedersdorf, Jack Keene lab, Duke University) we were able to access PAR-CLIP databases for known proteins that bind to the 3'UTR of the Cyclin D1 RNA (Figure 3.5). We then proceeded to determine if these genes were regulated by progestins by qPCR analysis. All the genes shown in Figure 3.6 were well expressed inT47D cells and AGO2 and FUS were both positive hits for PR responsiveness. However, it is important to note that several of these proteins can be modified at the post-translational level by kinases activated by mitogens. Thus one might not observe a change in the mRNA levels upon exposure to the mitogen, in this case progestins. Whether these genes are essential for PR-mediated Cyclin D1 instability remains to be investigated by siRNA screens.

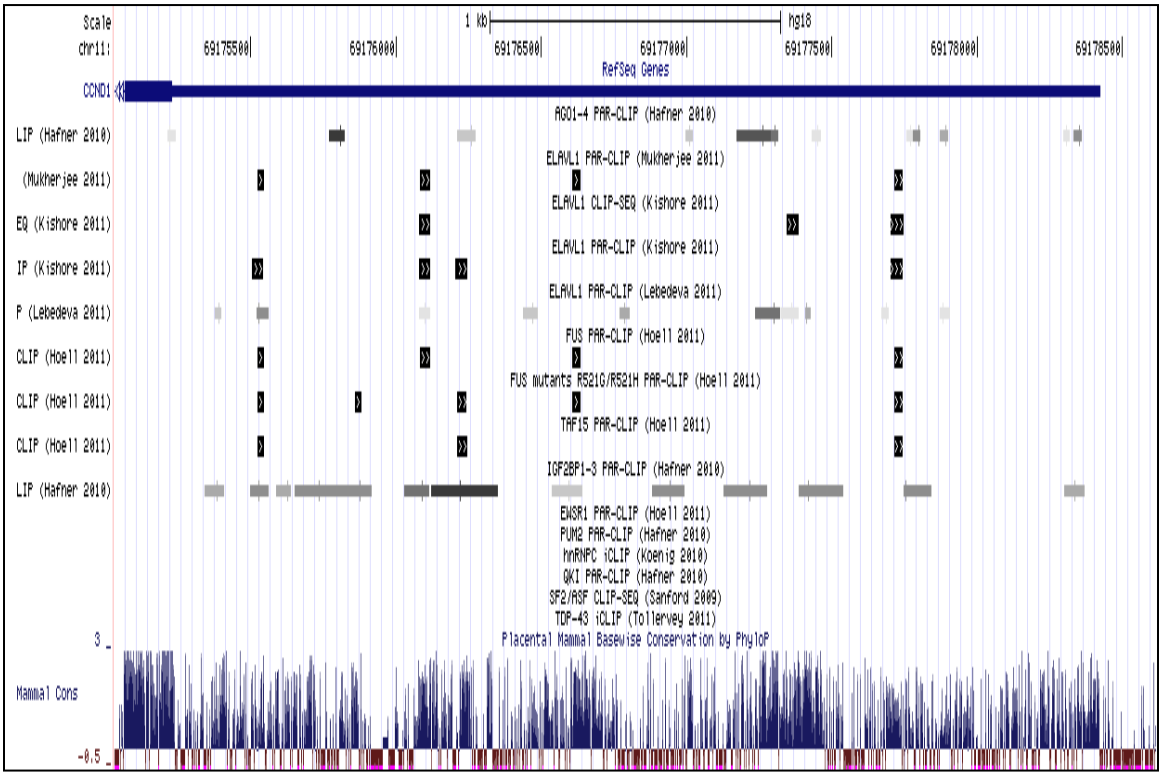


Figure 3.5: Identification of known RNA binding proteins that bind to the 3'UTR of Cyclin D1.

PAR-CLIP databases (scanned by the Keene lab, Duke University) were scanned to enlist all known proteins that bind to the 3'UTR of Cyclin D1. The 3'UTR is represented as a blue bar at the top of the figure. The left hand side indicates the assay performed in order to identify the RNA binding protein. The black boxes on each row indicate the binding sites on the 3'UTR for that protein. (produced by Matt Friedersdorf, Keene lab, Duke University, USA)

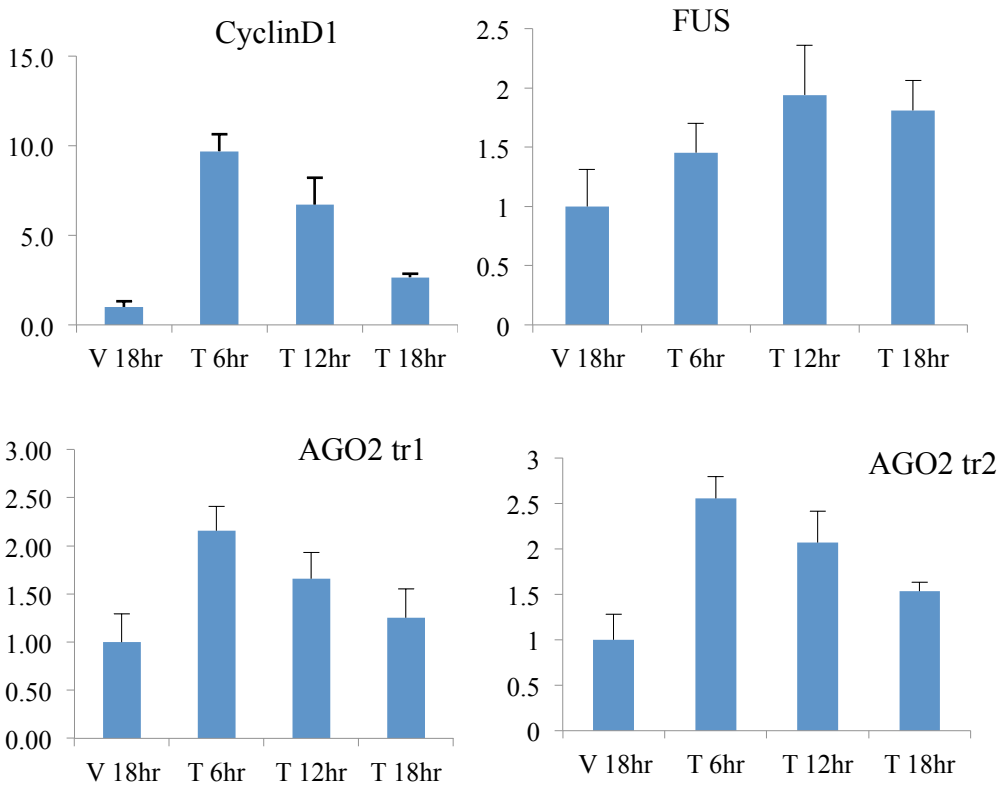


Figure 3.6: Progestins induce a sub-set of proteins that are known to bind to the 3'UTR of Cyclin D1.

Synchronized T47D:A18 cells were treated with Vehicle (V) or 100pM R5020 (T) for the time-points indicated. After treatment, cells were lysed and RNA was isolated and reverse transcribed. mRNA levels for individual genes were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM (n=3). AGO2 tr1 and AGO2 tr2 denote primers specific to transcript 1 and 2 of AGO2 respectively.

3.2.6 Analysis of the 3'UTR of Cyclin D1 by luciferase reporter assay

Since mRNA stability is typically regulated via the 3'UTR of the mRNA, we proceeded to clone the 3.2kB UTR of the Cyclin D1 gene at the end of the luciferase reporter gene into the pGL3 plasmid (pGL3-UTR) to establish a luciferase reporter-Cyclin D1-3'UTR plasmid. Figure 3.7 shows the basic experimental set-up, wherein we

propose to use the luciferase reporter as a surrogate to observe the activity of PR on Cyclin D1 3'UTR.

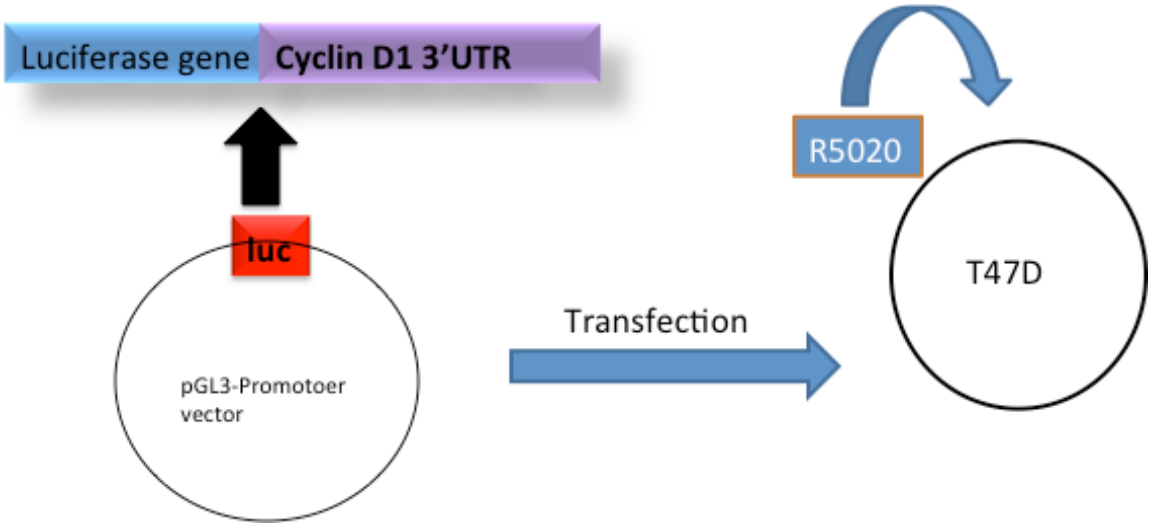


Figure 3.7: Experimental design- Transfection assay to determine role of the 3'UTR of Cyclin D1 in determining its mRNA stability.

Transient transfection of this Cyclin D1 3'UTR -luciferase reporter plasmid in T47D cells (Figure 3.8A) showed that the presence of the UTR reduced the basal level of luciferase expression irrespective of progestin treatment, implying that there are active sites of recruitment on the UTR for PR independent degradation proteins. Our initial hypothesis that PR decreases the stability of Cyclin D1 led us to predict that R5020 treatment would reduce the luciferase signal in the above assay. However, we were intrigued to discover that R5020 treatment actually increased the luciferase reporter activity containing the CCDN1 3'UTR. Similar results were also observed in HepG2 cells as well (data not shown).

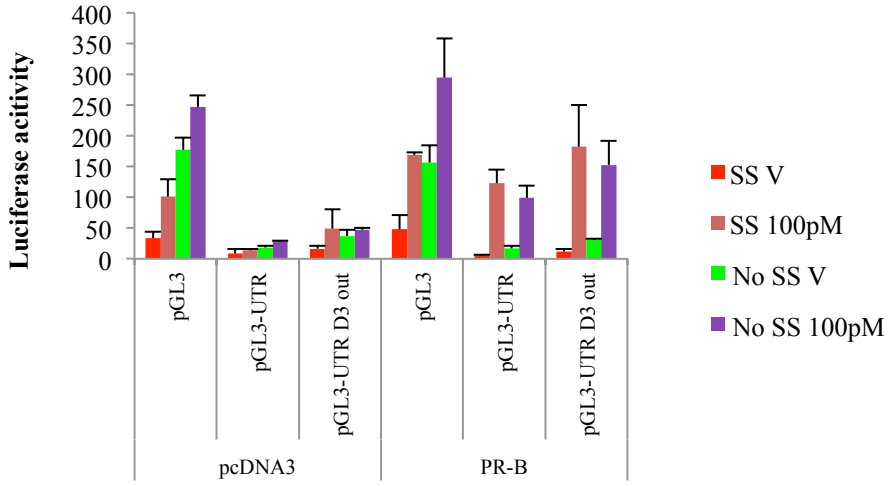
Since the enhancer element D3 was actually present on this UTR, we considered the possibility that D3, while having no activity as an isolated element, may function as an enhancer element in the presence of surrounding regions from the UTR. To address this possibility, we constructed a new luciferase plasmid called pGL3 D3-out, which had the 3'UTR with the D3 element removed. However this construct yielded the same results as the constructs with the full 3'UTR, implying that the absence of D3 did not affect the ability of PR to increase luciferase levels (Figure 3.8A).

In order to discern if the increased luciferase expression with the 3'CCDN1 UTR were because the experiments were not conducted under optimal conditions, we attempted to repeat the transfections with a different experimental set-up. Briefly, in (Figure 3.8A), we had transfected synchronized cells for 24hrs with the appropriate plasmids and then treated the cells with progestins for 24hrs. However, we speculated that the cells may need to be pre-treated with progestins before transfection to produce enough protein to act upon the luciferase reporter and destabilize it. To that end, we tested this (Figure 3.8B) by pre-treating synchronized or asynchronized cells with vehicle or R5020 for 12hrs prior to transfection. We included PR-A in order to ascertain whether this isoform may be playing a role in the regulation of the 3'UTR. For each condition, we also included a titration of the amount of reporter transfected onto the cells from 500ng to 1500ng.

We found that under all the conditions tested, pre-treating the cells yielded similar results to non-pretreated cells, implying that incubating the cells with progestins prior to introducing luciferase reporter with the 3' CCDN1 UTR does not affect PR regulation of

reporter expression levels. Figure 3.8B is a representative condition (1500ng reporter) from this experiment.

A)



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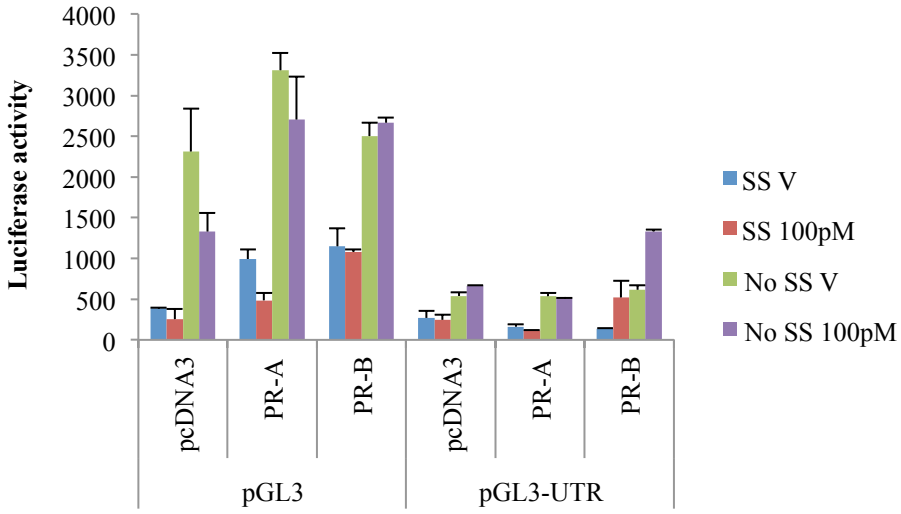

Figure 3.8: PR induces transcription of a luciferase-Cyclin D1 3'UTR reporter.

A) Synchronized (SS) or asynchronized (No SS) T47D:A18 cells were transfected with the indicated plasmids: i) pGL3 empty vector, or ii) pGL3-UTR- pGL3 plasmid which consisted of the Cyclin D1 3'UTR at the end of the luciferase reporter or iii) pGL3-UTR D3 out- pGL3 plasmid which consisted of Cyclin D1 3'UTR with the D3 element removed, at the end of the luciferase reporter, and pcDNA3 or PR-B expression plasmid. Cells were then treated with V or 100pM R5020 (100pM) for 48hrs and then lysed. B) Synchronized (SS) or asynchronized (No SS) T47D:A18 cells were treated with V or 100pM R5020 (100pM) for 11-12hrs followed by transfection with the indicated plasmids (pcDNA3, PR-A or PR-B expression plasmids and pGL3 empty vector or pGL3-UTR) for 24hrs and then lysed.
All wells were also co-transfected with pCMV- β gal. Cells were assayed for luciferase and β-galactosidase activity. Data are presented as normalized response obtained by normalizing luciferase activity with β-galactosidase activity.

The induction of luciferase by PR could mean that PR is i) inducing the transcription of the reporter gene by acting on a response element present on the UTR, or ii) PR is increasing the stability of the gene.

From the above data, we postulate that while progestin treatment of breast cancer cells leads to an eventual decrease in mRNA half-life that is concurrent with the cell cycle arrest induced by progestins, the regulation of Cyclin D1 mRNA in breast cancer is more complicated and a multimodal theory of regulation appears to be at work. Further experiments are warranted to explore other conditions and investigate the molecular pathways responsible for the results obtained from the above reporter assays.

3.3 Discussion

We have successfully established that over time, agonist-bound PR can directly or indirectly attenuate the half-life of accumulated Cyclin D1 mRNA. Overexpression studies showed that the candidate protein, TTP, was not the mediator of Cyclin D1 mRNA stability in our model system. However, TTP does play a role in the suppression

of the inflammatory response in breast cancer cells, which is a novel and exciting finding that warrants further investigation. We have also presented several potential candidate RNA binding proteins that are expressed in breast cancer cells and may play a role in the destabilization of Cyclin D1 mRNA upon steroid-activated cellular growth. Thus while the mechanism of the half-life reduction of PR-induced Cyclin D1 mRNA is still unclear, the data presented here bring forth some important questions, the resolution of which can guide the design of specific progesterone receptor modulators, or SPRMs, which will take advantage of the appropriate receptor conformation to enhance these pathways in PR positive breast cancers.

3.3.1 Cyclin D1 mRNA stability and disease

The clinical significance of Cyclin D1 mRNA instability is supported by several pieces of evidence. Lebwohl et al in 1994 found that a 1.1-1.3kB Cyclin D1 transcript that was truncated at the 3' end, was three fold amplified compared to the normal 4.2kB allele in MDA-MB 453 breast cancer cells (149). Moreover, this transcript also has a longer half-life than its full-length counterpart, thus implicating mRNA stability in the tumorigenesis of this cell line. In mantle cell lymphoma (MCL), a B cell lymphoma characterized by a specific gene translocation resulting in enhanced expression of Cyclin D1, two forms of the transcript are seen due to polyadenylation- the full length 4.4kB transcript and a 1.7kB shorter transcript. Studies have shown that the shorter form was lacking the 3'UTR and exhibited a longer half-life. The subset of tumors exhibiting elevated levels of the shorter form were also associated with higher levels of cell cycle

genes such as AURORA and TOP2A (150, 151). Rosenwald *et al* have shown that selective expression of short Cyclin D1 mRNA isoforms is a pathogenic mechanism that is associated with increased Cyclin D1 expression, increased proliferation, and shorter survival (152). Collectively, these data indicate that loss of the 3'UTR of the Cyclin D1 mRNA, resulting in a longer-lived transcript, has a significant correlation with tumor occurrence. Identification of proteins that prevent Cyclin D1 accumulation by decreasing its stability can be developed as a therapeutic target to down-regulate the levels of this oncogenic species in tumor pathogenesis.

3.3.2 Destabilization of Cyclin D1 mRNA upon cell cycle exit

Progestins have been shown to possess both proliferative and growth inhibitory roles in breast cancer. *In vitro*, progestin stimulation allows cells to go through one passage of the cell cycle and then arrest in G1. However, the cellular targets for this exit, followed by quiescence and inability to re-enter the cell cycle is still debated.

While widely established as an indispensable member of the G1-S transition, Cyclin D1 regulation is also critical for continuation of and exit from the cell cycle. While the addition of growth-factors stimulates initial Cyclin D1 production in a transcription dependent manner, in actively cycling cells, the fluctuation is controlled by post-transcriptional mechanisms. A study by Gao et al in 2005 found that upon forcing cells to exit the cell cycle via serum deprivation, Cyclin D1 mRNA is attenuated without any decline in its transcription rate (153). Furthermore, the mRNA exhibits a shorter half-

life when serum is removed, implicating Cyclin D1 message destabilization as a critical process in Cyclin D1 suppression in the withdrawal from the cell cycle

In T47D cells, treatment with the synthetic progestin ORG2058 results in the cells beginning to enter S phase between 8-10hrs, with maximal cells in S phase at 12hrs (95). In agreement with other independent studies (154), the length of stay in S-phase in this study was found to be 6hrs, which was followed by a rise in the percentage of cells in G2/M phase at 18hrs. T47D cells have a doubling time of 22hrs, which is again in alignment with the times at which they are seen to peak at G2/M phase (154). However, after 18hrs, proportion of G2/M phase cells continues to decline reaching a plateau by 33hrs (95). The expression pattern of Cyclin D1 in our T47D:A18 cells also show a well-paralleled timeline, wherein the levels peak by 6hrs just prior to entry of cells into S phase, followed by a decline from 12-24hrs when cells have completed mitosis and then remain quiescent. Neither Cyclin D1 levels (Figure 2.14 B, Chapter 2) nor the percentage of cells in S phase (95) shows any change after this decline. Considering that the Cyclin D1 transcript induced by progestins in our model system also showed a lower half-life at 18hrs as compared to 6hrs, we postulate that this decline in mRNA half-life in breast cancer cells possibly plays a vital role in the eventual arrest of these cells after one round of cellular replication upon progestin treatment.

Over-expression of Cyclin D1 without its 3'UTR that is thus potentially rendered degradation resistant, can help us evaluate whether this is sufficient to rescue cells from the progestin-induced G1 arrest. Moreover, further delineation of the factors that govern this destabilization will lead to knockdown experiments wherein we can determine the

validity of this hypothesis- i.e. can knockdown of this factor prevent the G1 arrest seen in T47D cells after one round of the cell cycle.

3.3.3 Inflammation and TTP in breast cancer.

We have shown evidence suggesting that TTP can suppress several target genes of the cytokine IL-1β mediated NF-κB inflammatory response in breast cancer. Several lines of evidence suggest that NF-κB plays an important role in mammary gland development and breast cancer (155). Briefly, NF-κB is a transcription factor complex that mediates several arms of the inflammatory response with target genes such as IL-6, IL-8, CCL2, CCL20 etc. The range of cellular processes regulated by this transcription factor extends beyond inflammation to anti-apoptotic and proliferatory pathways. Several studies have documented increased NF-κB DNA-binding activity in both breast cancer cell lines and primary human breast cancer tissues (156, 157). Rat mammary tumors induced by the carcinogen DMBA (7,12-dimethylbenzaanthracene) also exhibit elevated levels of nuclear NF-κB binding activity (158). Collectively, these data suggest that the inhibition of NF-κB offers a possible therapeutic option for breast cancer.

Thus, as a negative regulator of pro-inflammatory cytokines, TTP plays a role in tumor suppression by exerting inhibitory effects on several NF-κB gene targets and this was confirmed by data we have collected in our breast cancer model system as well. In fact, numerous independent pieces of evidence also underline an association of TTP with breast cancer progression, which we will summarize here. Suppression of TTP expression has been identified as a negative prognostic indicator in breast cancer and correlated with

higher tumor grade and adverse clinical outcome (159). TTP's anti-oncogenic function has also been linked to the oncogenic Ras pathway in a mast cell tumor model (160). Another study has demonstrated that the microRNA miR-29a mediates the suppression of TTP in coordination with Ras signaling (161). Recently discovered novel TTP targets in breast cancer such as MMP1, uPA, and uPAR have also been implicated in tumorigenic functions such as metastasis and invasion (162).

Taken together, these data highlight the important correlation of both TTP and NF-κB to breast cancer pathogenesis. The data indicates that the suppression of the NF-κB transcriptome by TTP is an important anti-oncogenic function in breast cancer that is of great therapeutic value. Therefore further delineation of the link between progestin induced TTP and the TTP-mediated inhibition of inflammatory genes in breast cancer will be of clinical significance and remains under investigation in our group.

In conclusion, our data highlights two important aspects of PR biology which warrant further investigation: i) The effects of PR on the stability of the pro-oncogenic Cyclin D1 mRNA- delineating the mechanism by which progestin treatment acts on the Cyclin D1 3'UTR and thus induces its destabilization could reveal therapeutic targets for breast cancer models, and ii) The role of the PR induced expression of the mRNA regulatory protein TTP in suppressing inflammatory genes. If our hypothesis that TTP plays a role in PR-mediated repression of the NF-κB inflammatory response is correct, the mechanistic information from these studies can be used to develop PR modulators that exhibit selectivity appropriate for breast cancer therapy.

4 Conclusions

Our studies on the PR regulation of Cyclin D1 were motivated by the discovery that the MAPK pathway might not be required for PR-induced hyperphosphorylation of the tumor suppressor Rb protein. Given the fact that Cyclin D1 activates the kinases Cdk 4/6 to phosphorylate Rb, we chose to focus on the PR/Cyclin D1/Rb axis for our studies. In this chapter we will discuss the significance of our findings from the perspective of the current literature, as well as our future plans for the further development of this work for the potential goal of developing PR as a therapeutic target.

The data surrounding PR regulation of Cyclin D1 expression is rather controversial. While some studies have suggested that PR is able to bind the Cyclin D1 gene through a tethering mechanism hence implying it does not occur through binding directly to a PRE (113, 114), others have shown recruitment to regions on the promoter but no functional data exists (112). The general consensus of the field has been that the interaction of PR with cytoplasmic signaling pathways, specifically the MAPK pathway, is required to achieve induction of Cyclin D1 levels. Studies have suggested that the interaction of PR with ERα is able to facilitate it's activation of MAPK (112), while others have proposed that PR can independently carry out the activation via the interaction of a polyproline motif in it's amino terminus with the SH3 domain of cSrc and thus activate the Ras/Raf/MAPK pathway (101).

However, in this study we have demonstrated that the primary role of progestins in the hyperphosphorylation of Rb is not the activation of the MAPK pathway but the direct genomic regulation of Cyclin D1. We have shown that progestins regulate Cyclin

D1 mRNA expression in a biphasic manner with maximal induction observed within 6hrs followed by a decrease in mRNA levels by 18hrs. Further analysis demonstrated that the progesterone receptor (PR) is recruited to a specific 16bp progesterone response element on the Cyclin D1 gene which is both necessary and sufficient for transcriptional activation by PR in a reporter assay. The functionality of the element was demonstrated by luciferase reporter assays wherein it was shown that the element could confer progesterone responsiveness to a heterologous promoter. Thus, contrary to what has been proposed by others, our data suggests that the Cyclin D1 gene is a primary target of PR.

It was also observed in the course of these studies that the stability of the Cyclin D1 mRNA is considerably different in cells treated with progestins for 18hrs as opposed to 6hrs; an activity which implies that progestins may limit the expression of Cyclin D1 mRNA by decreasing its stability. This is an interesting result in the context of the pattern of cell cycle regulation upon progestin treatment, wherein cells are able to complete a round of proliferation followed by arrest in G1(95). Studies have shown that when T47D cells are treated with the synthetic progestin ORG 2058, cells begin to enter S-phase by 8-10 hrs, followed by an eventual entry into G2/M phase by 18hrs and finally the completion of mitosis by 22hrs. This is highly synchronous with the pattern of Cyclin D1 expression upon progestin treatment, wherein high levels are achieved by 6hrs and levels decline by 18hrs at which point a majority of cells have made the transition to mitosis. We believe that our results can provide a possible explanation to the much debated effect of progestins in breast cancer cell proliferation. We hypothesize that progestins rapidly induce Cyclin D1 and its downstream effects to enable the cell to divide once. However,

progestins also exert negative effects on the Cyclin D1 gene such that its expression is attenuated around the time the cells approach G2/M phase, thus rendering the cells unable to re-divide. In order to explore this hypothesis, we believe it is essential to isolate the factor(s) that is responsible for the PR-mediated decrease in Cyclin D1 mRNA stability at 18hrs. We can then proceed to perform knockdown studies of this factor to determine if we are able to over-ride progestin-induced G1arrest after one round of the cell cycle is completed.

One of the reasons that the role of progestins in breast cancer is complex is it's seemingly paradoxical roles in breast cancer therapy. While progestins are used as third-line endocrine therapies in breast cancer, anti-progestins have also been shown to have varying degrees of success in breast cancer treatment. The progesterone receptor also exhibits both proliferative and anti-proliferative actions in cellular and animal models. For instance, while it is anti-proliferative in *in vitro* models such as MCF7 cells (163) as well as T47D cells, the latter cell line shows robust progestin-dependent growth in an *in vivo* mouse model (164). However this phenomenon can be explained in the light of data from Lange et al (165) wherein it was proposed that progestins can prime breast cancer cells for stimulation by growth factors such as epidermal growth factor. R5020 potentiates the effects of EGF by inducing EGFR, c-ErbB2 and c-ErbB3 receptors, and by increasing EGF-stimulated tyrosine phosphorylation of signaling molecules. Thus one can envision that progestins are growth stimulating in the microenvironment of a mouse tumor, which may have an interplay of hormones as well as growth factors.

It would be interesting to investigate whether the mRNA stability-modulating factor is suppressed in models of breast cancer wherein progestins are proliferative. If that is the case, then this can guide the development of new therapeutics to enhance or mimic the activity of this factor and thus stunt the growth of these breast cancer cells.

In the course of our studies on candidate proteins responsible for PR-mediated Cyclin D1 mRNA destabilization, we discovered that PR can induce the RNA binding protein tristetraprolin (TTP) at the RNA and protein level. While this protein was not important for Cyclin D1 half-life regulation, our overexpression studies demonstrated that it plays a role in the suppression of the IL-1β mediated inflammation pathway in breast cancer cells. This result is highly significant in the context of another aspect of progestin function involving PR-mediated suppression of inflammatory cytokines. Specifically, our lab has discovered that in breast cancer cells, IL-1β treatment robustly induces the expression of inflammatory cytokines through activation of the NF-κB pathway and that is abolished with progestin treatment (148). Our hypothesis is that PR induction of TTP plays a role in the suppression of the NF-κB pathway. Since our attempts at obtaining reliable siRNA against TTP were not successful, we propose the use of knockdown cell lines to address if these two phenomena are related, i.e is PR-induced suppression of NF-κB target genes mediated in part or fully by PR upregulation of TTP. The further study of the mechanism of PR-induction of TTP will enable a better understanding of the factors governing the entire pathway. Considering that very little is known about the role of PR in inflammation, these findings are novel and warrant further investigation. We can envision the development of PR modulators in the clinic that can

enhance the TTP-inducing activity of PR and thus work against inflammatory conditions such as cancer, fibroids and endometriosis.

In conclusion, the studies described in this thesis present us with a better understanding of the mechanisms of PR action in models of breast cancer. The further development of these projects will continue to elucidate further the complex role of PR in mediating breast cancer phenotypes, with the eventual goal of using this mechanistic information to refine existing PR modulators as well as to develop new drugs for the treatment of breast cancer.

5 Materials and methods

5.1 Biochemicals

Promegestone (R5020) was obtained from Perkin-Elmer (Waltham, MA. Catalog# NLP-004). 17β-estradiol (E2) (Catalog# E8875), dexamethasone (Dex) (Catalog# D4902), IL-1β (Catalog# I9401) and cycloheximide (CHX) (catalog# C7698) were obtained from Sigma-Aldrich (St. Louis, MO). U0126 (Catalog# S1102) and AZD6244 (Catalog# S1008) were obtained from Selleck Chemicals (Houston, TX). PD184161 and Actinomycin D were obtained from Calbiochem (SanDiego, CA). Human EGF and heregulin were purchased from Peprotech (Rocky Hill, NJ). PCR and qPCR reagents were obtained from Bio-Rad (Hercules, CA), and Integrated DNA Technologies (Coralville, IA).

5.2 Antibodies

Total Rb antibody was purchased from BD Pharmingen (San Jose, CA) (Catalog# 554136). Rabbit polyclonal antibodies against Total Erk (p42/p44 MAPK) and phospho-Rb Ser807/811 were from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody against Cyclin D1 was obtained from Neomarkers (Fremont, CA). Goat polyclonal antibodies against GAPDH (V-18) and mouse monoclonal antibody against P-Erk (phosphorylated p42/p44 MAPK) were obtained from Santacruz Biotechnology, Inc (Dallas, TX). TTP antibody was an affinity-purified antibody that was obtained from Sigma-Aldrich (St. Louis, MO) (Catalog# T5452).

The anti-mouse-HRP and anti-rabbit-HRP secondary antibody conjugates were obtained from Bio-rad. Anti-goat-HRP secondary antibody conjugate was purchased from Santacruz Biotechnology, Inc (Dallas, TX).

5.3 Expression plasmids

pcDNA3 was purchased from Invitrogen (Carlsbad, CA). pBKC-PR-B and pBKC-PR-A have been described before (166). PR-A and PR-B were constructed as follows: A BamH1 fragment of PR-B bearing amino acids 24-9333 was cut out from pBKC PR-B and inserted into pcDNA3 (PR-B 24-9333). PR-B was then amplified by PCR and then inserted at 5'end using Kpn-BstEII into pcDNA3 PRB 24-933, to create pcDNA3-hPR-B (PR-B). To create PR-A, a PR-A specific PCR product was inserted into PRB24-933 using KpnI-SrfI sites. The normalization vector pCMV-β-gal was obtained from Clontech (Palo Alto, CA). VP16 PR-B (pVP16-hPR-B) has been described previously (167).

A KpnI-EcoRI fragment from pcDNA3 PR-B or PR-A was cloned into pENTR-1A purchased from Invitrogen to create pENTR hPR-B and pENTR hPR-A respectively. Gal4DBD, PR-B, or PR-A was shuttled from pENTR-Gal4DBD, pENTR-hPR-B, or pENTR-hPR-A to an MSCV-IRES-EGFP vector that was converted to a Gateway destination vector, to create MSCV-GWb-Gal4DBD-IRES-EGFP, MSCV-GWb-hPR-B-IRES-EGFP, and MSCV-GWb-hPR-A- IRES-EGFP respectively. The Invitrogen Gateway recombinase subcloning system was used according to manufacturer's instructions for the above cloning.

The TTP expression vector was a kind gift from Dr.Jack Keene, (Duke University, Durham, NC). PR C587A was a kind gift from Dr.Horwitz (University of Colorado, Denver, CO) and has previously been described by her group (168)

5.4 Reporter constructs

pGL4.26 was purchased from Promega (Madison, WI). The plasmids D2, D3, and D4 were constructed by amplifying the D2, D3 and D4 regions respectively from T47D:A18 genomic DNA . D2 was amplified using the sense primer AGATCGGTACCGCACGTGCTCTCAGTGACTGTG and antisense primer AGATCGCTAGCGAATGAAAACCTTCTCATTGAG. D3 was amplified using the sense primer AGATCGGTACCTATTCTTTGCGTGTAGCTATGG and antisense primer AGATCGCTAGCGTTACATGTTGGTGCTGGgaag. D4 was amplified using the sense primer AGATCGGTACCGCCGTGACAACCAAGAATGTCT and antisense primer AGATCGCTAGCACCTGCAGCTGACAAGTAgcac. The amplified PCR products were then digested with Kpn1 and Nhe1 and cloned into pGL4.26 using Kpn1 and Nhe1 sites to create plasmids D2, D3 and D4. D2-PRE del was constructed by performing PCR-mediated deletion on the D2 plasmid with sense primer CCGTGGGAGGCCAGGTGAAAGGAGCTTCCGGGACAGC and antisense primer GCTGTCCCGGAAGCTCCTTTCACCTGGCCTCCCACGG. The consensus PRE plasmid (named PRE) was constructed by constructing a synthetic oligo with the 16bp PRE and sticky ends for Kpn1 and Nhe1 as follows. The sense strand had the sequence CGGAACGTCCAGTGCCG while antisense strand had the sequence

CTAGCGGCACTGGACGTTCCGGTAC. The oligo was annealed and directly ligated into the pGL4.26 vector that had been digested and linearized with Kpn1 and Nhe1. 1X PRE FKBP was a kind gift from Dr.John Norris (Duke University, Durham, NC). pGL3 plasmid was obtained from Promega (Madison, WI). 2XPRE-tk-Luc has been described previously (167). pGL3-UTR was constructed by amplifying the 3'UTR of Cyclin D1 using the sense primer AGATCTCTAGAAGGAGGAGGTGGACCTGGCTTGC and antisense primer AGATCTCTAGATCTAGACTTTCATGTTTGTCTTTTTGTC. The amplicon was digested with Xba1 and cloned into pGL3 using the Xba1 site. pGL3-UTR-D3 out was constructed using the pGL3-UTR plasmid as a template and the sense primer AGATCGAATTCTTATCCCCTGCCCCTT and antisense primer AGATCTCTAGAATCCCGAATGAGAGTCCTACA. The amplicon was digested with EcoR1 and Xba1 and cloned into the pGL3-UTR vector using EcoR1 and Xba1 sites. All plasmids were verified by sequencing.

5.5 Mammalian cell culture

The T47D:A18 cell line was kindly provided by V. Jordan (Fox Chase Cancer Center, Philadelphia, PA) and has been previously described (169). HepG2 cells, HeLa cells and and BT483 cells were purchased from the American Type Culture Collection (Manassas, VA). The PR-negative T47D:C42 cells were a kind gift from D. Edwards (Baylor College of Medicine, Houston, TX), and have been previously described (101).

T47D:C42-Gal4DBD, T47D:C42-hPR-B and T47D:C42-hPR-A stable cell lines were created as follows: parental T47D:C42 cells provided by Dean Edwards were

infected with MSCV-GWb-Gal4DBD-IRES-EGFP (negative control), MSCV-GWb-hPR-B-IRES-EGFP, or MSCV-GWb-hPR-B-C587A-IRES-EGFP expressed in a retroviral system. Enhanced green fluorescent protein (EGFP)-positive cells were then selected through two rounds of cell sorting using flow cytometry. PR expression was confirmed by qPCR (data not shown).

Unless otherwise noted, all media and supplements were purchased from Invitrogen. T47D:A18 and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 8% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 1mM Sodium Pyruvate (NaPyr) and 0.1mM Non essential amino acids (NEAA). HepG2 cells were maintained in BME (basal minimum essential) media (Sigma-Aldrich) supplemented with 8%FBS, 2mM L-glutamine, 1mM NaPyr and 0.1mM NEAA. BT483 cells were maintained in RPMI medium supplemented with 8% FBS, 1mM Sodium Pyruvate NaPyr and 0.1mM NEAA. T47D:C42 cells were maintained in Minimum Essential Medium (MEM) supplemented with 8% FBS, 10 mM HEPES, 25 μg/ml gentamicin, 50 U/mL Penicillin/Streptomycin (Pen/Strep), 0.1 mM NEAA and 1mM NaPyr . Cells were grown in a 37C incubator with 5% CO_2.

5.6 siRNA studies

Cells were seeded at a density of $0.25*10^6$ cells/well in 6-well plates, in phenol red-free DMEM containing 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr in the presence of 50 nM siRNA or siLuc (siRNA control) using DharmaFECT-1 (Dharmacon, Lafayette, CO) according to the manufacturer's recommendations for

reverse transfection of cells. Refer to table for siRNA sequences used. 48hrs later, the medium was switched to SS (serum starved) media, i.e. phenol red-free DMEM containing 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr for 24hrs. Cells were then treated with vehicle (ethanol) or R5020 as described in the figure and harvested for RNA or protein analysis after the appropriate time points.

Table 5.1: siRNA sequences

siRNA	**Sequence**
si Luciferase	Invitrogen Stealth RNAi proprietary sequence; Catalog #10620312
si CCND1 1	5'- UUGGAAAUGAACUUCACAUCUGUGG-3' Catalog# 10620319
si CCND1 2	5'-CCACAGAUGUGAAGUUCAUUUCCAA -3' Catalog# 10620318

5.7 RNA isolation and qPCR studies

Cells were seeded at a density of $0.25*10^6$ cells/well in 6-well plates in phenol red-free DMEM containing 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr. 48hrs later, media was changed to SS media (phenol red-free DMEM containing 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr) for 24hrs. Cells were then treated with the appropriate agonist for the indicated time points and harvested for RNA extraction. RNA was then extracted by using the Aurum^{TM} Total RNA Mini Kit

(Bio-Rad, Hercules, CA), 1µg of which was subject to cDNA synthesis by the iScript cDNA synthesis kit (Bio-rad). cDNA was then diluted 1:10 and 1.24uL was used along with 10uM primer mix per-well in a 384-well qPCR reaction performed in the Bio-Rad iCycler Realtime PCR System. Primer sequences used are listed in table.

Table 5.2: qPCR primers

Gene	qPCR Primer sequence
36B4	Forward: 5'- GGACATGTTGCTGGCCAATAA -3' Reverse: 5'- GGGCCCGAGACCAGTGTT -3'
CCND1	Forward: 5'-CCAGAGGCGGAGGAGAAC -3' Reverse: 5'- AAGCGTGTGAGGCGGTAG-3'
E2F1	Forward: 5'- ACGTGACGTGTCAGGACCT -3' Reverse: 5'- GATCGGGCCTTGTTTGCTCT -3'
S100P	Forward: 5'- AAGGTGCTGATGGAGAAGGAGC -3' Reverse: 5'- GCCACGAACACGATGAACTCAC -3'
FKBP51	Forward: 5'- CGGAGAACCAAACGGAAAGG -3' Reverse: 5'- CTTCGCCCACAGTGAATGC-3'
TTP	Forward: 5'- GCC AAC CGT TAC ACC ATG GAT CTG-3' Reverse: 5'- AAG TGG GTG AGG GTG ACA GCT CAG-3'
EGR1	Forward: 5'- GAGACCAGTTACCCCAGCC-3' Reverse: 5'- AAAGCGGCCAGTATAGGTGAT-3'
TNFα	Forward: 5'- ATGAGCACTGAAAGCATGATCC-3'

	Reverse: 5’-GAGGGCTGATTAGAGAGAGGTC -3’
CCL2	Forward: 5’- CAGCCAGATGCAATCAATGCC-3’ Reverse: 5’- TGGAATCCTGAACCCACTTCT-3’
CCL4	Forward: 5’- CTGTGCTGATCCCAGTGAATC-3’ Reverse: 5’- TCAGTTCAGTTCCAGGTCATACA-3’
CCL20	Forward: 5’- TGCTGTACCAAGAGTTTGCTC-3’ Reverse: 5’- CGCACACAGACAACTTTTTCTTT-3’
IL6	Forward: 5’- AACCTGAACCTTCCAAAGATGG -3’ Reverse: 5’- TCTGGCTTGTTCCTCACTACT -3’
IL8	Forward: 5’- TTTTGCCAAGGAGTGCTAAAGA-3’ Reverse: 5’- AACCCTCTGCACCCAGTTTTC-3’
COX2	Forward: 5’- GGCTTCCATTGACCAGAGCAG -3’ Reverse: 5’- GCCGAGGCTTTTCTACCAGA -3’
HIF1 α	Forward: 5’- GGACAGCCTCACCAAACAGA -3’ Reverse: 5’- GCAGTCTACATGCTAAATAATTCCT -3’
FUS	Forward: 5’- GGTACTCAGCGGTGTTGGAA-3’ Reverse: 5’-GTTCTGGCTCTGGCCATAAGA -3’
AGO2 tr1	Forward: 5’ CGCATCATCTTCTACCGCGA -3’ Reverse: 5’- CTTGTCCCCCGCTCGTTCTT -3’
AGO2 tr2	Forward: 5’- GTGGAAACATTCCAGCAGGC-3’ Reverse: 5’ - CCCAGAGGACGTGATAGTGC-3’

5.8 Chromatin immunoprecipitation assay

T47D:A18 cells were seeded in 15-cm dishes using DMEM supplemented with 8% FBS, 0.1 mM NEAA and 1 mM NaPyr for 24 h. Media was then switched to phenol red-free DMEM supplemented with 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr for 48 h, after which the cells were serum-starved for 24 hr in SS media (phenol red-free DMEM containing 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr). Following treatment with Vehicle or R5020 for the indicated time periods, cells were subjected to modified ChIP analysis as previously described (170) (102).

Table 5.3: ChIP Primers

Genomic region to be detected	qPCR primer
D1 set 1	Forward: 5'- TCCAGCAGTACAACCCGCCTTATT-3' Reverse: 5'-TCCGCGTTTACCGTAAGGCTTAGA-3'
D2 set 1	Forward: 5'-TGTGCACATGCTGCATACACTCAC-3' Reverse: 5'-TGTGCTTAGATTAGGAGCCACCCA-3'
D3 set 2	Forward: 5'-AGAACACGGCTCAGCTTACCTCAA-3' Reverse: 5'-TTGGCCTCTCGAACACACAACATC-3'
D4 set 2	Forward: 5'-CCGGGAGGCTCCAGAGAATAATTT-3' Reverse: 5'-ATCAATGGGCACCGCTCACGTC-3'
Stromelysin	Forward: 5'- TCTATCCCAAGCTGAAGAACTGGCCAGTCCCTGC -3'

	Reverse: 5’- CAAGTAGCTGGGACCACAGACGTGCGCCACCATG -3’
E2F1 Distal PRE #4 (E2F1 D4)	Forward: 5’- CCTGCAGGGTGCTAAGGATA -3’ Reverse: 5’- GCTTACTGGGCAAAGGTGAA -3’
E2F1 Distal PRE #5 (E2F1 D5)	Forward: 5’- AAATCAGACCACAGCAGCAG -3’ Reverse: 5’- TGGGTCCCTAAGCTCTGAGT -3’

5.9 Western blotting

T47D:A18 cells were seeded in 10cm plates at a density of $1.5*10^6$ cells/plate, or in 6-well plates at a density of $0.25*10^6$ cells/well in phenol red-free DMEM supplemented with 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr for 48 hrs. Cells were then serum starved for 24hrs in phenol red-free DMEM supplemented with 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr.

HeLa cells were seeded overnight in 6-well plates in DMEM supplemented with 8% FBS, 0.1 mM NEAA and 1 mM NaPyr, following which they were treated with EGF to a final concentration of 20ng/mL for 2hrs.

After treatment with the appropriate ligands, cells were scraped in ice-cold PBS. After centrifugation for 5mins at 4°C , the cell pellet was lysed in RIPA Buffer [50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 50 mM NaF, 2 mM Na_3VO_4, and 1X protease inhibitor mixture (EMD Chemicals, Inc, San Diego, CA)] while rotating at 4°C for 30 min. Resultant protein was

loaded onto 7.5% poly-acrylamide gels and resolved by SDS-PAGE, transferred onto a PVDF membrane (Bio-Rad) and blotted with the appropriate antibodies.

5.10 Transient transfection assays

5.10.1 Reporter assays

For 96-well luciferase reporter assays, T47D:A18 cells were seeded at a density of 10,000 cells/well in 96-well cell culture plates or 60,000 cells/well in 24-well cell culture plates, in phenol red-free DMEM supplemented with 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr. Cells were transfected with Fugene (Promega, Madison, WI) according to manufacturer's instructions. HepG2 cells were seeded at a density of 18,000 cells/well in phenol-red free MEM supplemented with 8% charcoal stripped serum, 2mM L-glutamine, 1mM NaPyr, and 0.1mM NEAA. Cells were transfected with Lipofectin (Invitrogen) according to the manufacturer's protocol.

Briefly, for a 96-well plate, each transfection was performed in triplicate using 550ng of total DNA. Each transfection, contained 500ng of luciferase reporter, 25ng of pcDNA3, PR-A or PR-B, and 25ng of pCMV-β-Gal to normalize for transfection efficiency. 24-well plates had a total of 3000ng of DNA per triplicate. Transfection of this DNA into cells was allowed to proceed for 24hr. Depending on the assay and as indicated in the figure descriptions, the cells were serum starved for 24hrs (T47D:A18 cells- phenol red-free DMEM supplemented with 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr. HepG2 cells- phenol-red free BME media with 0.1% charcoal

stripped serum, 2mM L-glutamine, 1mM NaPyr, and 0.1mM NEAA) after the transfection. At this time a 24-48hr incubation with hormones was initiated.

5.10.2 TTP over-expression assays

T47D:A18 cells were seeded overnight in 10cm plates at a density of $1.5*10^6$ cells/well in phenol red-free DMEM supplemented with 8% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr. The next day, for each well, 294uL OPTI-MEM was combined with 24uL Fugene (Promega, Madison, WI). The solution was mixed well and left at room temperature for 5mins. In another tube 8ug DNA was diluted in OPTI-MEM to make up a total volume of 80uL. The OPTI-MEM and DNA mix were added to the Fugene solution and incubated at 37C for 15mins. The mix was added drop wise to the cells. Cells were serum starved by the addition of SS media (phenol red-free DMEM containing 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr) the next day for a period of 24hrs. Appropriate ligands were then added and cells were harvested for RNA or protein.

HeLa cells were seeded overnight in 10-cm plates and transfected the next day with 8ug plasmid according to the above protocol. EGF was added to a final concentration of 20ng/mL after 24hrs of serum starvation (DMEM containing 0.1% FBS, 0.1 mM NEAA and 1 mM NaPyr) and cells were collected for RNA after 2hrs.

5.11 Actinomycin D RNA-stabilty assay

T47D:A18 cells were seeded at a density of $0.25*10^6$ cells/well in 6-well plates in phenol red-free DMEM supplemented with 8% charcoal stripped FBS, 0.1 mM NEAA

and 1 mM NaPyr. 48hrs later, media was changed to SS media (phenol red-free DMEM containing 0.1% charcoal stripped FBS, 0.1 mM NEAA and 1 mM NaPyr). After 24hrs in SS media, appropriate ligands were added. After a period of 6hrs or 18hrs, 100ng/uL of Actinomycin D (ActD) was added to the cells to inhibit transcription. Cells were then collected after every hour for a period up to 4hrs for RNA. RNA was extracted using the Aurum^{TM} Total RNA Mini Kit (Bio-Rad, Hercules, CA), 1ug of which was subject to cDNA synthesis by the iScript cDNA synthesis kit (Bio-rad). cDNA was then diluted 1:10 and 1.24uL was used along with 10uM primer mix per-well in a 384-well qPCR reaction performed in the Bio-Rad iCycler Realtime PCR System.

6 Appendix: *In vitro* characterization of the 5α reduced progesterone metabolite, 5α dihydroprogesterone

6.1 Introduction

For decades, the fundamental principle underlying mammalian reproductive biology has been the requirement of progesterone for the establishment and maintenance of pregnancy (8, 171). The progesterone released from the corpus luteum in the luteal phase of the menstrual cycle prepares the uterine bed for a potential implantation. Once fertilization occurs, the placenta is able to establish a constant release of progesterone that maintains the uterine environment for the growing embryo. In fact the end of pregnancy is signaled by a withdrawal of progesterone levels (172), leading to uterine contractions during labor.

Thus to date, apart from progesterone, no other bioactive endogenous pregnane that can support pregnancy has been identified in mammals. However, interestingly, in some mammals such as mares, elephants or the rock hyrax, the levels of circulating progesterone during pregnancy are extremely low and not sufficient to sustain pregnancy (173-176). While the existence for alternate endogenous progestins have been hypothesized, the data supporting the identity and functionality of these alternative pregnanes are debatable (177, 178) (179) (180).

6.2 Results

The 5α reduced metabolites of progesterone such as 5α dihydroprogesterone (DHP) are very highly expressed in pregnant mares (181, 182). Using a combination of *in vitro* and *in vivo* approaches, Scholtz et al have shown that DHP is an endogenous

biopotent progestin that is capable of maintaining pregnancy in mares in the absence of progesterone, thus establishing it as the first endogenous pregnane other than progesterone to play a vital role in mammalian reproduction (183). Specifically, this study showed that endometrial biopsies of ovariectomized mares treated with DHP showed glandular development and expression of progesterone target genes such as uterocalin (P19) to levels similar to that seen in the progestational luteal phase in cyclic mares. Using prostaglandin F2α to stimulate luteolysis thereby causing a regression of luteal progesterone secretion, they found that DHP was able to maintain healthy embryonic development (seven out of nine mares) until day 27 after establishment of pregnancy. In contrast, the vehicle treated mares were unable to sustain their pregnancy. Thus while our collaborators had determined that DHP is important for maintaining pregnancy in mares, whether it was functioning as a PR ligand was not clear. Thus we set out to characterize this equine progestin using *in vitro* assays.

6.2.1 DHP is as potent as progesterone for the equine PR

In order to perform the luciferase reporter assays, HepG2 cells were transfected with ePR, cloned from an equine uterine cDNA library, or hPR-B. We chose hPR-B since it exhibits a greater degree of similarity to the ePR amino acid sequence, and the immunoblot analysis in Figure 6.1 demonstrated that ePR was similar in length to hPR-B and not hPR-A.

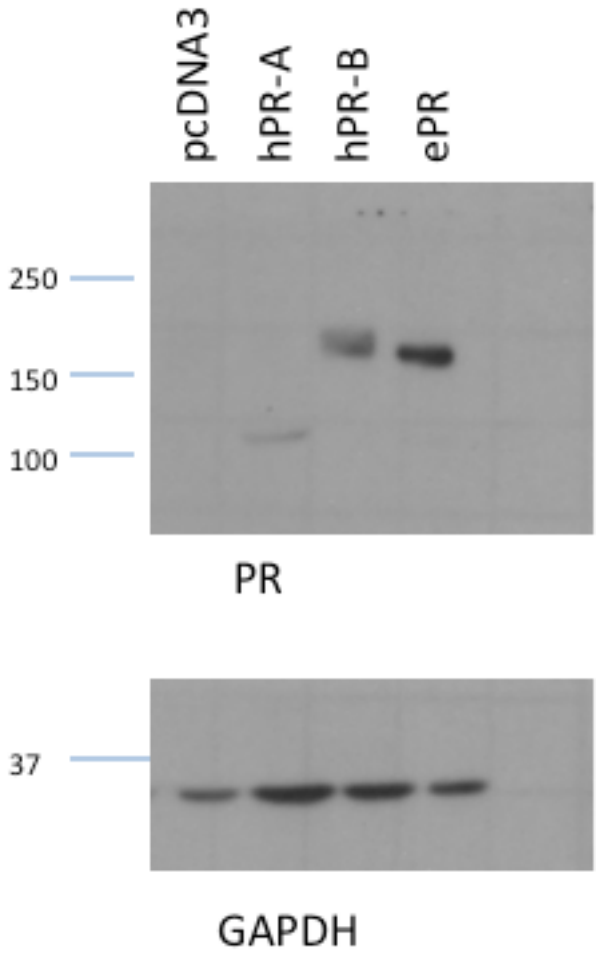

Figure 6.1: – Immunoblot showing expression levels of hPR and ePR (183).

Immunoblot analysis of human progesterone receptors (hPR-A and hPR-B) and equine progesterone receptor (ePR). HepG2 cells were transfected with the indicated plasmid for 24hrs. Cells were then harvested; 18 μg of whole-cell extract was resolved by SDS/PAGE, transferred to PVDF, and subjected to immunoblotting for progesterone receptor (PR) or GAPDH (loading control).

Cells expressing ePR were treated with DHP or progesterone as a comparison, with a parallel set of treatments with the two hormones for hPR as well (Figure 6.2). EC50 calculations were based on the efficacy curves plotted with the luciferase activity against a range of hormone treatments (0.15nM-1uM). Both DHP and progesterone manifested similar half maximal concentrations for ePR (EC50 = 14+/- 1.1nM and 13.7 +/- 1.6nM respectively). These concentrations were relevant in the physiological context since circulating DHP levels by the 12th week of pregnancy are twice the EC50 of DHP for ePR (183). However the affinity of DHP for human PR was about 20% that of progesterone.

In conclusion, we have demonstrated that the endogenous pregnane DHP can activate the ePR as robustly as progesterone. Our collaborators showed that DHP is able to induce the expression of target genes relevant for the maintenance of pregnancy in mare uterus and support embryo growth in the absence of detectable progesterone. Together, these studies provide direct evidence that DHP is as strong a progestin as progesterone with respect to ePR and that it is in fact the progestin that maintains pregnancy in mares in the face of almost undetectable levels of progesterone itself.

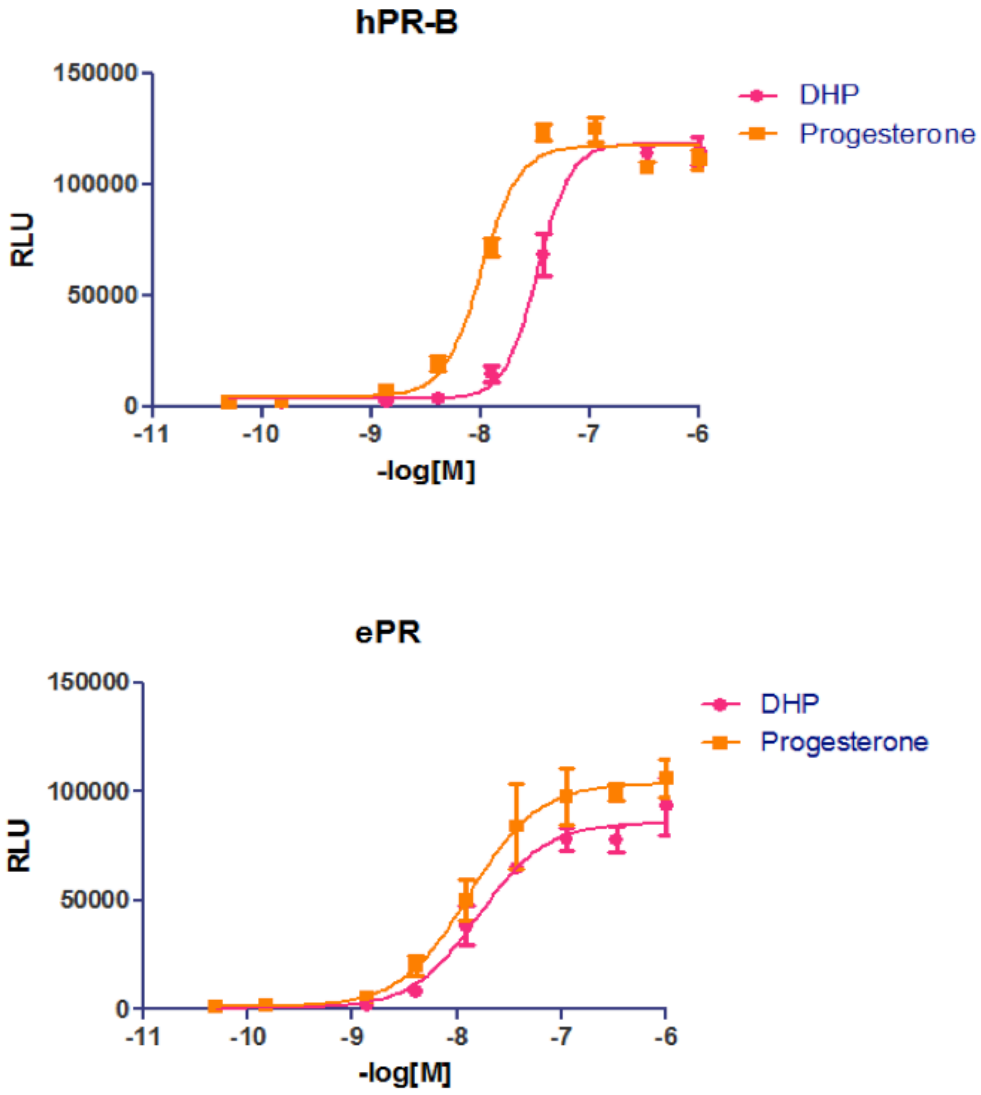

Figure 6.2: DHP activates ePR as potently as progesterone (183).

HepG2 cells were transfected for 24hr with ePR or PR-B, pCMV-β-Gal, and MMTV-luc (reporter). Cells were then induced for 24 hr with 5alfa DHP or progesterone (as indicated) and assayed for luciferase and β-galactosidase activity. Data are presented as normalized response, which was obtained by normalizing luciferase activity with β-galactosidase activity. Error presented as ±SD of triplicate points.

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Biography

Shweta Krishnan
Date of birth: October 2nd, 1986
Place of birth: Hyderabad, Andhra Pradesh, India

Education:

2004 – 2008: Bachelor's of Science (Molecular Genetics), University of Rochester

2008 – 2014: Doctoral Program in Pharmacology and Cancer biology, Laboratory of Dr. Donald P. McDonnell, Duke University

Publications: Scholtz EL, Krishnan S, Ball BA, Corbin CJ, Moeller BC, Stanley SD, et al. Pregnancy without progesterone in horses defines a second endogenous biopotent progesterone receptor agonist, 5alpha-dihydroprogesterone. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(9):3365-70.

Fellowships:

2008-2009: Chancellor's Fellowship, Duke University

2010-2013: Department of Defense (DOD) Breast Cancer Research Program (BCRP) Predoctoral Award W81XWH-11-1-0063.