Multimodal Regulation of Gene Transcription by Progestins

by

Hilary Erin Wade

Department of Pharmacology and Cancer Biology
Duke University

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Donald McDonnell, Supervisor

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Anthony Means

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Tso-Pang Yao

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Kenneth Korach

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
of Duke University

2009
ABSTRACT

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Abstract

The progesterone receptor (PR) is a member of the nuclear receptor superfamily of ligand-regulated transcription factors. The steroid hormone progesterone binds to PR and induces a conformational change that enables the receptor to bind DNA, recruit cofactors, and directly regulate the transcription of target genes. In addition, extra-nuclear PR can indirectly regulate gene expression by rapidly activating other signaling pathways such as Src/MAPK. Although the direct and indirect functions of PR have been well studied in isolation, it is important to understand the molecular mechanisms by which these pathways can cross talk and integrate to ultimately impact gene expression.

Towards this end, we initiated studies to assess the overall impact of MAPK inhibition on PR transcriptional activity in T47D breast cancer cells treated with the synthetic progestin R5020. During the course of microarray and biochemical analyses that were undertaken to address this issue, we discovered a subset of PR target genes that are enriched for E2F binding sites. Subsequently, we determined that PR-B is a component of several distinct pathways that function both directly and indirectly to positively up-regulate E2F1 expression in T47D breast cancer cells. Firstly, PR directly regulates E2F1 transcription by binding to proximal and distal enhancer sites located near E2F1. Secondly, progestin induces the hyperphosphorylation of Rb, which results in increased recruitment of E2F1 to its own promoter, thereby activating a positive feedback loop that further amplifies its transcription. Finally, PR induces expression of Krüppel-like factor 15 (KLF15) and potentially other Sp/KLF family members, which can bind to
GC-rich DNA within the E2F1 promoter and further activate transcription. Together, these results suggest a paradigm for multimodal PR gene regulation that entails cooperation between direct and indirect pathways of PR signaling to achieve the desired downstream transcriptional cascade.

In the breast and other tissues of the female reproductive system, progesterone plays an important role in normal development and function. Therefore, synthetic PR modulators (PRMs) are widely used to manipulate the downstream biology of PR for purposes including contraception and hormone replacement therapy (HRT). However, progestins and PR have also been implicated in disease pathologies such as breast cancer. While the molecular mechanisms by which PR regulates breast tumor growth have not been fully elucidated, recent studies highlight the fact that progestins may have a dose-dependent role in breast cancer progression. Consequently, we undertook studies to identify and characterize any differential effects of low-dose versus high-dose progestins on the downstream activities of PR. Specifically, we found that treatment of breast cancer cells with low-dose progestins can induce maximal transcriptional activation of a subset of PR target genes, including the cell cycle regulators cyclin D1 and E2F1. Furthermore, low-dose and high-dose progestins have differential effects on the phosphorylation of PR and subsequent receptor turnover. Cumulatively, these findings underscore the importance of establishing the effects of a wide range of progestin concentrations on target gene expression and other PR actions, so that we are able to accurately predict the potential consequences of PRMs on downstream PR signaling pathways and biology.
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<td>17-OH-preg</td>
<td>17-hydroxy-pregnenolone</td>
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<tr>
<td>17-OH-prog</td>
<td>17-hydroxy-progesterone</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ActD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>AF (-1, -2, -3)</td>
<td>Activation function</td>
</tr>
<tr>
<td>AIB1</td>
<td>Amplified in breast cancer-1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BUS</td>
<td>B-upstream segment</td>
</tr>
<tr>
<td>C</td>
<td>Carboxy (-terminus)</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARM1</td>
<td>Coactivator-associated arginine methyltransferase 1</td>
</tr>
<tr>
<td>CBP/p300</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CDC (2 and 6)</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>CDK (2 and 4)</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CEE</td>
<td>Conjugated equine estrogens</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>---------------------------------------</td>
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<tr>
<td>CHX</td>
<td>Cycloheximide</td>
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<td>CKII</td>
<td>Casein kinase II</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CoA</td>
<td>Coactivator</td>
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<tr>
<td>CoAA</td>
<td>Coactivator activator</td>
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<tr>
<td>CS-FBS</td>
<td>Charcoal-stripped fetal bovine serum</td>
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<td>CTE</td>
<td>Carboxy terminal extension</td>
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<td>CYP11A</td>
<td>Cytochrome P450 11A</td>
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<td>DNA binding domain</td>
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<td>Dex</td>
<td>Dexamethasone</td>
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<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E2</td>
<td>17β-estradiol</td>
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<tr>
<td>E6-AP</td>
<td>Ubiquitin-protein ligase E6-associated protein</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>ER (α and β)</td>
<td>Estrogen receptor</td>
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<table>
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<td>ERID</td>
<td>ER-interacting domain</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FKB51</td>
<td>FK506-binding immunophilin 51</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GO-term</td>
<td>Gene ontology term</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GRE</td>
<td>Glucocorticoid-responsive element</td>
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<td>h</td>
<td>Hinge region</td>
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<td>HABITS</td>
<td>Hormonal replacement therapy after breast cancer: is it safe?</td>
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<td>HAT</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HMGB</td>
<td>High mobility group protein</td>
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<td>Hormone response element</td>
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<td>Inner mitochondrial membrane</td>
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<td>Internal ribosome entry site</td>
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<td>JAK</td>
<td>Janus family of tyrosine kinases</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<td>KLF</td>
<td>Krüppel-like factor</td>
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<td>MAPK phosphatase 1</td>
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<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>MPA</td>
<td>Medroxy progesterone acetate</td>
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mRNA  Messenger ribonucleic acid
N  Amino (-terminus)
NaPyr  Sodium pyruvate
NEAA  Non-essential amino acids
NFκB  Nuclear factor kappa B
NLS  Nuclear localization signal
NR  Nuclear receptor
P4  Pregn-4-ene-3,20-dione (or progesterone)
PBS  Phosphate buffered saline
p/CIP  p300/CBP interacting protein
pCAF  p300/CBP-associated factor
PCR  Polymerase chain reaction
Pen/Strep  Penicillin/streptomycin
PEPCK  Phosphoenolpyruvate carboxykinase
PI3K  Phosphotidylinositol (PI) 3-kinase
PIAS  Protein inhibitor of activated STAT
PR  Progesterone receptor;  h=human,  c=chicken,  x=Xenopus,  m=membrane,  g=genomic
PRE  Progesterone response element
PRM  Progesterone receptor modulator
PRMT1  Protein arginine methyltransferase 1
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<td>Quantitative real time polymerase chain reaction</td>
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</tr>
<tr>
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<td>Serine</td>
</tr>
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</tr>
<tr>
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<td>Src homology 3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Smad</td>
<td>Sma- and Mad-related protein</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid or thyroid hormone receptor</td>
</tr>
<tr>
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<td>Specificity protein 1</td>
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<td>Synthesis phase</td>
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<td>Steroid receptor coactivator-3</td>
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<tr>
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<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
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<td>Small ubiquitin-related modifier</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian vaculolating virus 40</td>
</tr>
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1 Background

1.1 Thesis Research

The principle goal of my thesis work was to achieve a better understanding of progesterone receptor (PR) actions in breast cancer. Through these studies, we aimed to uncover new ways in which progestins and PR regulate downstream biology through control of target gene transcription and cross talk with other signaling pathways.

1.2 Progesterone Receptor Signaling

1.2.1 Progesterone

1.2.1.1 Biosynthesis

Progesterone, also known as P4 (pregn-4-ene-3,20-dione), is a naturally occurring steroid hormone that functions as an endogenous agonist for PR. In females, the biosynthesis of progesterone occurs primarily in the ovaries, but progesterone is also synthesized in the placenta during pregnancy, and by the adrenal cortex (79). Physiological levels of progesterone fluctuate dramatically throughout the typical 28-day female reproductive cycle (Figure 1.1). During the initial follicular stage, circulating progesterone levels are extremely low (less than 2 ng/mL), and estrogen is the predominant steroid hormone produced in the ovaries (209). The transition from the follicular phase to the luteal phase of the reproductive cycle is characterized by a dramatic surge in levels of pituitary luteinizing hormone (LH), which stimulates an
increase in the biosynthesis of progesterone. Circulating progesterone levels during the luteal phase range from 2 to 20 ng/mL (209).

![Graph of Female Reproductive Cycle](image)

**Figure 1.1: Female reproductive cycle**

A graph depicting relative blood hormone levels during each phase of the 28-day female reproductive cycle.

As with all steroid hormones, the *de novo* synthesis of progesterone begins with the transport of cholesterol from the cytoplasm to the inner mitochondrial membrane (IMM), a process that is facilitated by the steroidogenic acute regulatory (StAR) protein (131). At the IMM, the side-chain cleavage enzyme cytochrome P450 11A (CYP11A) catalyzes the conversion of cholesterol to pregnenolone (Figure 1.2) (118). Next, pregnenolone is transported to the endoplasmic reticulum, where it is converted to progesterone by the actions of 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone then serves as a “precursor” hormone that is further metabolized into other
steroid hormones, including mineralcorticoids, glucocorticoids, androgens and estrogens (Figure 1.3) (22).

Figure 1.2: Progesterone biogenesis

A diagram of the reactions in the steroidogenic pathway that contribute to the synthesis of endogenous progesterone, highlighting the enzymes that catalyze these reactions. CYP11A (cytochrome P450 11A), 3β-HSD (3beta-hydroxysteroid dehydrogenase).

Figure 1.3: Steroidogenesis

1.2.1.2 Physiological Roles

Progesterone plays a central role in the development, growth and differentiation of the female reproductive system, and is required for the establishment and maintenance of pregnancy (for review, see (30)). During the female menstrual cycle, progesterone mediates ovulation by stimulating rupture of the ovarian follicle, and prepares the uterus for successful implantation by antagonizing the proliferative actions of estrogen and stimulating the decidualization of uterine stromal cells. Throughout pregnancy, progesterone promotes uterine quiescence by preventing the uterine myometrium from undergoing contractions. Furthermore, progesterone aids in the pregnancy-induced maturation of the mammary secretory gland by stimulating ductal branching and lobuloalveolar differentiation, but acts to repress milk secretion until after parturition (reviewed in (73)). The multiple roles of progesterone in female reproductive biology have led to the development of many synthetic PR modulators (PRMs) over the past few decades, which are widely prescribed to manipulate these downstream PR actions for purposes including contraception, termination of pregnancy, and hormone replacement therapy (HRT).

However, it is important to note that while progesterone is predominantly categorized as a female sex hormone, this view is somewhat misleading, since biosynthesis of progesterone also occurs in the Leydig cells of the testes and in the adrenal cortex of men (79). In fact, serum progesterone levels in males are quite similar to those seen in females during the follicular phase of the menstrual cycle (132). While
the biological functions of progestins and PR in male physiology remain significantly less defined, studies suggest roles in male-typical sexual behaviors and sperm function (9, 139). Furthermore, progesterone and its metabolites have been implicated in many non-reproductive processes in both sexes, including regulation of the cardiovascular system, immune responses, the central nervous system, and bone (30).

1.2.2 Progesterone receptor

1.2.2.1 PR-A and PR-B

The biological functions of progesterone are primarily mediated by the two PR isoforms, PR-A and PR-B. In both chickens and humans, a single gene encodes the two PR isoforms. The chicken PR (cPR) isoforms arise by alternative initiation of translation from two separate start codons located within a single mRNA transcript (28). However, the two human PR (hPR) isoforms are produced from two unique mRNA transcripts, which are generated through the utilization of two distinct estrogen-regulated promoters within the PGR gene (81).

1.2.2.1.1 Modular domain structure

PR-A and PR-B are relatively close in size, with respective molecular weights of 94 and 114 kDa. Structurally, the principle difference between the two human PR isoforms is that PR-B contains an additional N-terminal stretch of 164 amino acids, termed the B-upstream segment (BUS), which is lacking in PR-A. Otherwise, the hPR isoforms share the same modular structure that is characteristic of nuclear receptors (Figure 1.4).
Figure 1.4: PR structure

Schematic illustration of characteristic NR modular structure, with specific domain structure of hPR isoforms. N (amino-terminus), AF (activation function), BUS (B-upstream segment), ID (inhibitory domain), DBD (DNA-binding domain), h (hinge region), LBD (ligand-binding domain), C (carboxy-terminus).

The poorly conserved N-terminal A/B domain includes the first of two activation function domains (AF-1), which are responsible for the transcriptional activation of target genes (58, 114). The extra N-terminal segment that is unique to PR-B contains a putative third AF domain (AF-3) (160). The central C domain encompasses the highly conserved zinc-finger DNA-binding domain (DBD), which contains sequences that dictate the specificity of DNA binding and also contribute to receptor dimerization (42). Immediately flanking the DBD on the right is a hypervariable hinge region (h) within the D domain, which contains nuclear localization signals (60). Further downstream, the C-terminal E/F domain houses the functionally complex ligand-binding domain (LBD),
which contains the second AF domain (AF-2) (113). In addition to ligand binding, the LBD includes sequences that enable the interaction of the inactive receptor with heat shock proteins and which are involved in receptor dimerization (29).

1.2.2.1.2 Localization

While both PR-A and PR-B are co-expressed in reproductive tissues, the relative ratio of the individual isoforms varies throughout development and the female menstrual cycle (104, 119). The normal ratio of PR-A:B has been found to be dysregulated in pathological processes such as carcinogenesis, suggesting that the ratio of PR isoforms may be important for regulating the biological functions of progestins (56). Furthermore, the localization of each PR isoform differs at the cellular level. Interestingly, studies with green fluorescent protein (GFP)-tagged PR have revealed that PR-A and PR-B exhibit distinct intracellular distributions; specifically, unliganded PR-B is located primarily in the cytoplasm, whereas unliganded PR-A exists predominantly in the nucleus of mammalian cells (92).

1.2.2.1.3 Molecular activities

In vitro studies have established beyond a doubt that PR-A and PR-B exhibit very different transactivation properties, depending on the cell type and promoter context (reviewed in (48). In general, PR-B can function as a much stronger activator of transcription in contexts where PR-A is weak or even inactive (114). An N-terminal inhibitory domain (ID) was identified that suppresses the transcriptional activity of PR-A; the ID is present in PR-B, but is not inhibitory, due to constraints imposed by the BUS
Furthermore, the BUS domain confers upon PR-B the ability to recruit a subset of co-activators that are not efficiently recruited by agonist-bound PR-A (47). Thus, differential coactivator recruitment is one explanation for the observation that PR-A exhibits weaker transcriptional activity than PR-B.

In addition, PR-A can act as a dominant repressor of PR-B transcriptional activity (196). PR-A displays a higher affinity for corepressors such as silencing mediator for retinoid or thyroid-hormone receptor (SMRT), and this ID-mediated interaction may facilitate PR-A transrepression of PR-B function (47). Moreover, PR-A can also function to transrepress gene activation by the estrogen, androgen, glucocorticoid and mineralocorticoid receptors (108). The mechanism of PR-A transrepression has been linked to N-terminal sumoylation and intramolecular communication, and will be further discussed in a subsequent section (2). Both PR isoforms act to transrepress ER activity in the presence of antagonists (85, 86, 107). However, the two PR isoforms do exhibit different responses to progestin antagonists; specifically, although antagonist-bound PR-A is inactive, antagonist-bound PR-B can be switched to a strongly active transcription factor in the presence of cyclic adenosine monophosphate (cAMP) (159) (12, 161).

Finally, the differential molecular activities of PR-A and PR-B translate to a corresponding difference in the regulation of downstream target gene expression by each PR isoform. As expected, recent large-scale studies on global PR gene regulation in human T47D breast cancer cells and Ishikawa endometrial cancer cells expressing either
PR-A or PR-B alone have confirmed that the two PR isoforms regulate different subsets of genes in the presence of progestins (150, 172).

1.2.2.1.4 Biological functions

In vivo determination of the functional differences between PR-A and PR-B at the physiological level has largely been derived from genetic studies in mice (reviewed in (30, 31)). PR expression is required for female fertility, and ablation of both PR isoforms results in pleiotropic reproductive abnormalities, including failure to ovulate, uterine hyperplasia and inflammation, impaired mammary gland alveolar development, and loss of appropriate sexual behavior (99). Moreover, isoform-specific PR knockout mouse models have demonstrated that PR-A and PR-B act in a tissue-specific manner to mediate the reproductive functions of progesterone. Mice lacking PR-A are infertile and display severe abnormalities in ovarian and uterine function, but exhibit normal mammary gland and thymus development (121). Conversely, selective ablation of PR-B causes a reduction in pregnancy-associated mammary ductal and alveolar morphogenesis, but does not affect ovarian, uterine or thymic responses to progesterone (120). Thus, PR-A is both necessary and sufficient to induce the progesterone-dependent reproductive responses that are required for female fertility, whereas the actions of PR-B are essential for normal proliferative responses of the mammary gland to progesterone.
1.2.2.2 Other PR variants

1.2.2.2.1 PR-C

A putative third PR isoform, PR-C, has been reported in several studies. Wei et al identified a 60-kDa progestin-binding protein in T47D breast cancer cells that resides in the cytoplasm in the absence of ligand, but translocates to the nucleus following treatment with progestins (206). PR-C is a N-terminally truncated form of PR that may arise from unique mRNA transcripts; while it lacks the first zinc finger of the DBD, it possesses a complete LBD and has been shown to enhance progestin-induced transcriptional activity (204, 205). PR-C has been identified in several human tissues, including decidual cells, fetal membranes and the placenta (54, 187). However, the existence of a third PR isoform remains somewhat controversial, and some studies have concluded that PR-C is not a naturally occurring PR isoform (158).

1.2.2.2 Membrane-associated receptors

Progesterone and other steroid hormones can exert rapid, non-genomic actions in cells that appear to lack the classical genomic PR (gPR), and this has led to an increasing number of studies that explore the potential actions of steroids at the cell surface membrane (reviewed in (130, 142, 201, 203)). Putative membrane-associated progestin receptors (mPR) have been isolated and cloned from a variety of tissues in multiple species, but the potential existence of progestin-specific, membrane-associated receptors has been most heavily researched in the Xenopus oocyte and in human spermatozoa. For example, progesterone actions that stimulate resumption of meiosis and maturation of the
Xenopus oocytes are thought to be mediated by a G protein-coupled receptor at the plasma membrane that differs from intracellular PR-A or PR-B (reviewed in (101)). Other studies have proposed that a plasma membrane-associated receptor is responsible for mediating the progesterone-induced acrosome reaction in human spermatozoa (155). Moreover, three putative mPR isoforms (α, β and γ) have been identified in humans, and they display distinct tissue-selective expression patterns in reproductive, neural, kidney and intestinal tissues (221).

However, the existence and mechanisms of action of membrane-associated steroid hormone receptors remain fairly controversial. For example, several recent studies have concluded that a Xenopus homolog of the traditional nuclear PR (xPR) may mediate the effects of progesterone on oocyte maturation (11, 191). Inconsistencies have also been noted regarding the human model of mPR action; for instance, gPR mRNA has been identified in human spermatozoa, which were previously thought to lack intracellular gPR (18). Further research is necessary to obtain a better understanding of rapid, non-genomic progesterone signaling pathways and the putative involvement of membrane-associated receptors in these processes.

1.2.3 Molecular mechanisms of PR action

1.2.3.1 Classical PR functions

PR is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors [for review, see (90)]. The classical pathway of PR transcriptional activation is depicted in Figure 1.5. Briefly, in the absence of ligand, PR is sequestered
by heat shock proteins (HSP) and maintained in an inactive state in the cytoplasm of target cells. Upon ligand binding, PR undergoes a conformational change that leads to its dissociation from the heat shock protein complex, an event that facilitates receptor dimerization and translocation into the nucleus. The receptor dimer is then capable of interacting with specific progesterone responsive elements (PREs) within target gene promoters. The DNA-bound receptor subsequently nucleates the assembly of large cofactor-containing protein complexes that can either positively or negatively impact gene transcription.

**Figure 1.5: Classical pathway of PR transcriptional activation**

Model depicting classic, nuclear functions of PR that stimulate transcription of target genes. HSP (heat shock protein), PRE (progesterone response element), CoA (coactivator).
1.2.3.1.1 Interaction with HSP

Steroid hormones such as progesterone circulate throughout the body and elicit the appropriate biological actions by binding to intracellular receptors, which function as transcription factors to modulate downstream gene expression. Therefore, it is essential that a receptor be able to exist in one of two states: an inactive or an active form. The inactive form of PR is sequestered in the cytoplasm in a chaperone complex including members of the HSP family and immunophilins (reviewed in (24, 143)). In the early days of steroid receptor research, many groups had established that receptors exist in two forms of different sizes: a small 4S form, and a large 9S form (reviewed in (77)). The larger 9S PR form was later discovered to be a cytosolic receptor-HSP heterocomplex (21, 165). Studies in chickens demonstrated that HSP90 interacts with the LBD of cPR (20, 163). The LBD of PR consists of twelve α-helices that are closely folded to form a characteristic 3-layer “helical sandwich” that forms the hydrophobic ligand-binding pocket (87, 210). Interestingly, the interaction between HSP90 and the LBD of PR facilitates a high-affinity steroid-binding conformation of PR (175).

1.2.3.1.2 Ligand binding and conformational change

Exposure to progesterone allows PR to switch to an active state. Association of hormone with the hydrophobic ligand binding pocket induces a conformational change in PR, whereby the multi-helix fold of the LBD closes around the ligand, and the C-terminal helix 12 acts as a lid to enclose the hormone-binding pocket (4, 202, 207, 210). This conformational change has many functional consequences, beginning with the
dissociation of ligand-bound PR from the HSP chaperone complexes (36). After its release from chaperone complexes, ligand-bound PR is then able to migrate to the nucleus; this transport is facilitated by nuclear localization signals (NLS), located within regions spanning the second zinc finger of the DBD, the hinge region and the LBD (60, 213). In a recent study, Graham et al identified a specific consensus motif within PR that acts as a nuclear-matrix targeting signal; specifically, they demonstrated that mutation of two adjacent leucine residues at positions 690-691 was able to completely abolish transport of PR to the nuclear matrix (55).

1.2.3.1.3 Dimerization and DNA binding

After translocation to the nucleus, ligand-bound PR forms homodimers that bind cooperatively to PREs within the promoter region of target genes (51). Multiple interfaces contribute to the homodimerization process, including the D-box within the DBD, regions located in the hinge domain and LBD, and additional N-terminal sequences (188). Ligand-bound PR homodimers recognize and bind to DNA in a sequence-specific manner (91). The glucocorticoid, androgen, progesterone and mineralocorticoid receptors have been shown to bind to a consensus hormone response element (HRE) consisting of a semi-palindromic inverted repeat of the hexanucleotide 5′-TGTTCT-3′ half-site separated by a three base-pair spacer (Figure 1.6) (reviewed in (44, 46)). Recent studies have computed a position weight matrix (PWM) for PR binding sites; these analyses revealed that the nucleotides in the right half-site are much more highly conserved than those in the left-half site (180, 181).
The consensus core HRE sequence, consisting of two hexanucleotide half-sites arranged as a semi-palindromic inverted repeat, where n (in 3-bp spacer) is any amino acid.

The tethering of PR to DNA is mediated by the N-terminal DBD, which is the most highly conserved (and perhaps the best-characterized) domain of nuclear receptors in general (reviewed in (42, 87, 222)). The typical NR DBD can be divided into two main regions: a highly conserved globular domain that serves as the core DBD, and a less conserved carboxyl-terminal extension (CTE). The core DBD consists of two extended, asymmetric zinc-binding modules, commonly known as zinc fingers, which each contain four invariant cysteine residues that coordinate binding of a zinc ion (43). The actions of the zinc fingers result in the formation and stabilization of two perpendicular $\alpha$-helices that interact specifically with consensus DNA sequences. The first (or N-terminal) helix inserts into the major groove of the HRE, making base-specific contacts that dictate sequence-specific binding (194). The second (or C-terminal) helix then lies perpendicular across helix 1 to create a compact hydrophobic core. The previously mentioned D-box, or dimerization domain, is located within helix 2 in a short loop between the first and second cysteines of the second zinc finger (149). Interactions between the respective D-box domains of each PR monomer allow the receptors to homodimerize and bind head-to-head on adjacent hexanucleotide half-sites. The 3-bp spacer region ensures that the
centers of each binding site are separated by 9 base pairs, or approximately one turn of the DNA helix; as a result, both DBD monomers are able to bind to the same surface of the DNA duplex (97, 166).

In contrast to the core DBD, the CTE is much less conserved in both structure and sequence; moreover, little is known about the potential contribution of the CTE to the DNA binding function of steroid receptors. The crystal structure of the PR DBD-CTE-DNA complex was recently solved, and it was found to be very similar to that of other nuclear receptors (151). Interestingly, Roemer et al determined that the PR CTE made additional contacts with DNA regions outside of the canonical HRE; specifically, Arg\textsuperscript{637} and Lys\textsuperscript{638} of the CTE were observed to contact the DNA minor groove at sites in the flanking regions around the PRE. The DBD was also demonstrated to interact with the narrow minor groove in the 3-bp spacer DNA region. Although it has been established that PR, GR, MR and AR can all bind to the same consensus HRE, they regulate different sets of target genes. One possible explanation for this selectivity could be differential cofactor recruitment; however, the aforementioned study suggests that sequence preferences in the three-bp spacer and flanking DNA regions may allow PR to bind and activate certain target genes with greater affinity.

In addition to regulating HRE selection and DNA binding, the DBD of PR can also facilitate certain protein interactions that alter receptor activity. For instance, high mobility group (HMGB) proteins are chromatin proteins that possess no sequence specificity, but bind preferentially to bent DNA; moreover, HMGB proteins are endowed
with architectural capabilities that allow them to bend DNA in order to help transcription factors bind to their cognate sites (reviewed in (3, 19, 189)). HMGB 1 and 2 have been shown to act as coregulatory proteins for all steroid receptors by enhancing sequence-specific binding to HREs; moreover, the CTE of PR is required for interaction with HMGB 1 and 2, and for efficient, high-affinity binding of PR to HRE half-sites (16, 111, 112, 134, 153, 198, 199, 215).

1.2.3.1.4 Ligand-dependent transactivation

Once ligand-bound PR homodimers are associated with a PRE within the target gene promoter region, they can enhance transcription through interactions with steroid receptor coactivators (reviewed in (110)). Ligand-dependent recruitment of coactivators is mediated largely through the AF-2 domain with the LBD. The previously mentioned agonist-induced LBD conformational change, particularly the rearrangement of helix 12, creates a hydrophobic cleft that facilitates coactivator binding to PR (41). The AF-2 cleft interacts with an amphipathic helix that is conserved on the surface of certain coactivators: the LXXLL motif (where L is leucine and X is any amino acid), also termed the NR box (63). Interestingly, the BUS in PR-B also contains two consensus LXXLL motifs that have been implicated in AF-3 function (192). Finally, in the presence of antagonists, the PR AF-2 hydrophobic pocket mediates interactions with corepressor proteins through a similar domain, known as a CoRNR box motif (173).
1.2.3.1.5 Coactivator actions

Nuclear receptor coactivators can enhance the rate of PR target gene transcription through a diverse variety of mechanisms (reviewed in (90)). Several PR coactivators contain histone acetyltransferase (HAT) activities, including steroid receptor coactivator-1, -2 and -3 (members of the SRC/p160 family); thyroid hormone receptor activating protein of 220kDa (TRAP220); CREB-binding protein (CBP/p300); and p300/CBP-associated factor (pCAF) (69, 74, 133, 148, 174, 177, 220). PR also recruits methyltransferases such as coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) (23, 84). Modification of histone tails around the target gene promoter and transcriptional start site is thought to facilitate access of basal transcriptional machinery to the promoter (25, 178).

PR also recruits coactivators with functions that are not involved in transcription initiation; for example, a growing body of evidence suggests that nuclear receptors can affect mRNA elongation, processing and transport (173). For instance, PR can modulate RNA splicing through interaction with coregulators such as coactivator activator (CoAA) (5, 6). In addition, PR interacts with members of the ubiquitin-proteasome pathway, which can lead to receptor turnover, but also stimulate the transcriptional activity of PR; this will be discussed in further detail below.

Finally, the ability of PR to associate with certain coactivators can provide an alternative mechanism by which PR can modulate transcription without binding directly to DNA. For example, several groups have demonstrated that PR can stimulate
transcription of target genes with promoter regions that lack a canonical PRE, but instead contain GC-rich binding sites for members of the specificity protein (Sp) family; in some cases, this stimulation of promoter activity appears to result from interactions between PR and Sp1 that lead to indirect tethering of the receptor to the target gene promoter (45, 137, 156, 168). Similarly, PR may regulate target gene transcription by tethering to other DNA-bound transcription factors, such as activating protein 1 (AP-1) or nuclear factor kappa B (NFκB) (52).

1.2.3.2 Rapid, extranuclear PR signaling

An accumulating body of evidence indicates that direct receptor regulation of target gene expression is not the only pathway by which PR can modulate downstream signal transduction. Ligand-activated extranuclear PR can trigger rapid effects on a diverse array of cellular signaling cascades in a manner that does not require the classical nuclear actions of PR. Thus far, treatment with progestins have been shown to rapidly activate the Src/Ras/mitogen-activated protein kinase (MAPK), the phosphoinositol 3-kinase (PI3K)/Akt/NFκB and the Janus family of tyrosine kinases (JAK)/signal transducer and activator of transcription (STAT) signaling pathways in breast cancer cells (14, 144, 157).

Progestin activation of the Src/MAPK cascade is perhaps the best studied of the extranuclear actions of PR; however, the mechanism by which PR regulates the Src/MAPK signaling cascade remains somewhat controversial. Edwards and coworkers have reported that the N-terminal domain of PR contains a polyproline motif (aa 421-
428) that can mediate rapid, direct interaction of progestin-bound PR with the SH3 domains of c-Src family tyrosine kinases (17). This direct physical interaction between PR and Src results in displacement of an autoinhibitory intramolecular SH3 domain interaction, which subsequently leads to activation of Src kinases and downstream MAPK.

In contrast, other groups have proposed an alternative mechanism whereby rapid progestin activation of Src/MAPK occurs via cross talk with ER (116). Previous studies have established that estrogens can rapidly activate the Src/MAPK pathway through an interaction of ERα with the SH2 domain of c-Src (115). However, recent work by Ballare and coworkers indicates that ligand-free ER also plays a role in rapid progestin activation of Src/MAPK (10). Specifically, they identified two ER-interacting domains (ERID-I and –II) within the N-terminal region of PR-B that mediate a direct interaction between ligand-activated PR and unliganded ERα in breast cancer cells. Thus, treatment with progestins facilitates the formation of an intracellular complex between the two nuclear receptors, which in turn allows ER to rapidly activate the Src/MAPK signaling cascade.

### 1.2.3.3 Integration of nuclear and extranuclear PR actions

PR can function both as a nuclear transcription factor and as an extranuclear modulator of cell signaling cascades; however, these two pathways of PR action do not operate in isolation. In fact, previous studies have established that the cross talk between PR and cytoplasmic signal transduction pathways is intricate and bidirectional (141). On one hand, PR can activate rapid extranuclear signaling pathways such as that regulated by
Src/MAPK and thereby modulate MAPK-dependent transcription. Conversely, kinases that have been rapidly activated by PR can reciprocate by altering the post-translational modification of PR or its coactivators. For instance, phosphorylation of PR can modulate its localization, relative expression levels, or ability to directly regulate target gene transcription; this will be discussed in further detail in a later section.

Phosphorylation of cofactors can also have a dramatic impact on the transcriptional program set in motion by nuclear receptors. For instance, it was recently reported that phosphorylation of steroid receptor coactivator-3 (SRC-3; also known as ACTR, AIB1, p/CIP, RAC3, or TRAM-1) can affect not only its activity, but different patterns of phosphorylation on SRC-3 can dictate the specificity of SRC-3 for various transcription factors (212). Although not yet studied in detail, it is likely, by extrapolation from studies of other NRs, that cofactor phosphorylation will also have a dramatic effect on PR transcriptional activity in cells. Cumulatively, studies highlighting the importance of the cross talk that occurs between the nuclear and extranuclear functions of PR have provided the impetus to define the molecular mechanisms by which these pathways can interact and integrate to ultimately impact gene expression.

1.2.4 Post-translational modification of PR

In the absence of ligand, PR-A and PR-B have respective molecular weights of 94 and 114 kDa; however, treatment with progestins results in an increase in the apparent molecular weight of both PR-A and PR-B (70, 93). This characteristic decrease (or upward shift) in electrophoretic mobility of PR on sodium dodecyl sulfate (SDS) gels
after treatment with progestins has been linked to post-translational modifications of the receptor such as phosphorylation (170).

1.2.4.1 Phosphorylation

![Figure 1.7: Sites of PR phosphorylation](image)

Schematic illustration of PR structure highlighting the location of twelve phosphorylation sites. With the exception of threonine 430 (labeled in red), phospho-sites are serine residues. Hormone-dependent phosphorylation sites are labeled in blue. N (amino-terminus), Thr (threonine), DBD (DNA-binding domain), h (hinge region), LBD (ligand-binding domain), C (carboxy-terminus).

As shown in Figure 1.7, PR can be phosphorylated on several different serine (Ser) and threonine (Thr) residues; most of these sites are located within the N-terminal domain, although one site of PR phosphorylation has been identified within the C-terminal hinge region (83, 216-218). Ser$^{81}$, Ser$^{102}$ and Ser$^{162}$ are unique to PR-B, as these sites are located within the N-terminal segment that is missing in PR-A; conversely, the remaining phosphorylation sites are located within regions shared by PR-A and PR-B. Interestingly, although Ser$^{294}$ is present in both PR isoforms, it appears to be preferentially phosphorylated on PR-B (27). Edwards and coworkers speculate that the
distinct conformation of the N-terminal domains of PR-A may inhibit phosphorylation of this site.

The phosphorylation of PR is a multi-step process that can be divided into three separate stages (183). Studies in T47D breast cancer cells have shown that PR is basally phosphorylated in the absence of hormone, with vehicle-treated cells exhibiting approximately 50% maximal PR phosphorylation (13). This first stage of basal PR phosphorylation occurs on at least nine sites: Ser$^{81}$, Ser$^{162}$, Ser$^{190}$, Ser$^{202}$, Ser$^{213}$, Ser$^{400}$, Ser$^{554}$, Ser$^{676}$ and Thr$^{430}$. In the second stage of PR phosphorylation, treatment with progestins results in a rapid increase in phosphorylation at the aforementioned basally phosphorylated sites, which occurs within five to ten minutes post-treatment. This rapid phase of hormone-inducible phosphorylation does not appear to be dependent on binding of PR to DNA (185). Finally, within one to two hours after treatment with progestins, phosphorylation of PR occurs at several hormone-dependent sites, including Ser$^{102}$, Ser$^{294}$ and Ser$^{345}$. No basal phosphorylation is observed at these sites in the absence of ligand; in fact, the third stage of hormone-dependent phosphorylation requires ligand-dependent binding of PR to its cognate DNA response element, and results in the characteristic upshift in PR mobility on SDS gels (7, 185, 217).

Previous studies have demonstrated that PR can be phosphorylated by a variety of kinases. For example, Ser$^{81}$ is located within a consensus sequence for casein kinase II (CKII), and in vitro studies show that CKII preferentially phosphorylates Ser$^{81}$, despite the existence of eleven other putative CKII sites within PR (218). Except for Ser$^{81}$, the
majority of PR phosphorylation sites identified thus far occur within Ser-Pro motifs, which indicates that proline-directed kinases, including mitogen-activated protein kinases (MAPKs) or cyclin-dependent kinases (Cdks), may be largely responsible for the phosphorylation of PR (217). Indeed, in vitro studies have shown that Cyclin A-cyclin-dependent kinase 2 (CDK2), in complex with cyclin A, can phosphorylate PR at Ser\textsuperscript{162}, Ser\textsuperscript{190}, Ser\textsuperscript{213}, Ser\textsuperscript{400}, Ser\textsuperscript{554}, Ser\textsuperscript{676} and Thr\textsuperscript{430} (83, 216). In contrast, Ser\textsuperscript{294} and Ser\textsuperscript{345} have been shown to be targets of the MAPK signaling pathway (89, 129, 169, 216, 217).

To date, the functional roles of each PR phosphorylation event remain largely undefined. In general, mutation of PR phosphorylation sites has resulted in little or no effects on receptor transactivation. While mutations of Ser\textsuperscript{190}, Ser\textsuperscript{554}, or Ser\textsuperscript{676} have been shown to slightly decrease PR transactivation, the mechanism(s) behind these effects has not been identified (184). Phosphorylation of PR on Ser\textsuperscript{345} has been linked to the characteristic upward shift in electrophoretic mobility of PR on SDS gels after treatment with progestins; however, this ligand-dependent decrease in PR mobility does not appear to affect the transcriptional activity of PR, as a mutant lacking the upshift (due to mutation of Ser\textsuperscript{345}) has been shown to be fully active (170, 184).

Perhaps the most well-characterized phospho-site in terms of PR function is Ser\textsuperscript{294}, a hormone-dependent MAPK phosphorylation site. Previous studies have established that phosphorylation of Ser\textsuperscript{294} can regulate the subcellular localization of PR; specifically, phosphorylation of PR on Ser\textsuperscript{294} by MAPK results in the rapid nuclear translocation of unliganded PR (146). Moreover, Ser\textsuperscript{294} is located within a nine-amino-
acid motif called a destruction box, and recent studies have demonstrated that phosphorylation status of PR at Ser\(^{294}\) plays an essential role in receptor turnover (89, 169). Specifically, treatment of T47D cells with R5020 stimulates PR phosphorylation by MAPKs on Ser\(^{294}\), which targets PR for subsequent ubiquitination and rapid degradation of PR via the 26S proteasome pathway (89). This ligand-dependent receptor turnover can be blocked by treatment with proteasome inhibitors, such as lactacystin and calpain inhibitor I; by mutation of Ser\(^{294}\) to alanine; or by specific inhibition of p42/44 MAPK activity, which abolishes phosphorylation of PR at Ser\(^{294}\) (89, 169).

Paradoxically, the receptor turnover induced by MAPK phosphorylation of PR at Ser\(^{294}\) has been linked to increased efficiency of PR as a transcriptional activator (169). Amplification of MAPK activity, either through over-expression of constitutively active MAPK kinase kinase 1 (MEKK1), or pre-treatment with epidermal growth factor (EGF), results in increased transcriptional activation by PR after treatment with progestins. However, mutation of Ser\(^{294}\) to alanine, which abrogates receptor turnover, also results in a loss of progestin synergy with EGF, and a decrease in PR transactivation. These data suggest that the ability of progestins to hyperactivate downstream gene transcription via cross talk with other rapid signaling pathways, such as MAPK, is functionally coupled to receptor degradation.

1.2.4.2 Ubiquitination

PR can serve as a substrate for ubiquitination, a three-step process whereby ubiquitin is activated by an E1 ubiquitin-activating enzyme and covalently linked to
lysine residues of substrate proteins by E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (64). Poly-ubiquitination usually targets a protein for subsequent degradation by the 26S proteasome. Indeed, as discussed above, phosphorylation of PR on Ser\textsuperscript{294} leads to the formation of PR-ubiquitin conjugates and subsequently results in proteasome-dependent receptor turnover (89).

There is also some evidence to support a role for the ubiquitin pathway in co-activation of PR transcriptional activity. Both the yeast E3 ubiquitin ligase RSP5 and its human homolog hRPF1 have been shown to increase the transcriptional activity of hormone-bound PR in mammalian cells (72). Furthermore, the ubiquitin-conjugating enzyme UBCH7 is recruited to PR-responsive promoters in a hormone-dependent manner, and can act in concert with the ubiquitin-protein ligase E6-associated protein (E6-AP) to synergistically enhance PR transactivation (197).

1.2.4.3 Sumoylation

PR function can also be modified by sumoylation, which involves the covalent linkage of small ubiquitin-related modifier (SUMO) to lysine residues on the substrate protein (40). As previously described for ubiquitination, sumoylation involves a similar three-step process that incorporates the actions of an E1 activating enzyme, an E2 conjugation enzyme, and an E3 ligase (62). A SUMO-1 consensus binding motif, $\psi$KXE (where $\psi$ is hydrophobic and $X$ is any residue), was recently identified within the N-terminal domain of PR at lysine 388 (2, 186). In fact, the N-terminal inhibitory function of PR is linked to sumoylation at Lys\textsuperscript{388}, as mutation of this site has been shown to
relieve auto-inhibition of PR; furthermore, sumoylation at Lys$^{388}$ also mediates the transrepressive actions of PR (2).

Sumoylation of PR at Lys$^{388}$ involves the E3 ligase activity of protein inhibitor of activated STAT-1 (PIAS1) or PIAS3 (80, 103). While the attachment of SUMO to PR occurs primarily at Lys$^{388}$, over-expression of PIAS3 can also stimulate sumoylation of PR-B at Lys$^7$ and Lys$^{531}$ (103). Recent studies have demonstrated that rapid sumoylation of PR at Lys$^{388}$ after treatment with progestins leads to repression of PR transcriptional activity and delays hormone-dependent receptor turnover (33). Furthermore, phosphorylation of PR at Ser$^{294}$ was shown to block sumoylation of PR and thereby de-repress receptor activity. Interestingly, mutation of Lys$^{388}$ to arginine resulted in a PR mutant that cannot be sumoylated and is hypersensitive to low progestin concentrations.

1.2.4.4 Acetylation

Finally, acetylation has been shown to modulate the transcriptional activities of several nuclear receptors, including the androgen receptor (AR) and ER (53). While a conserved KXXK motif has been identified within the hinge region of PR at aa 638-641, PR regulation by acetylation remains to be confirmed (40). One piece of data that supports a possible role for acetylation in modulation of PR function is that two proteins linked to de-acetylation, template-activating factor Ibeta (TAF-Iβ) and pp32, have been shown to bind to PR in pull-down assays (95, 96).
1.3 Breast Cancer

1.3.1 General information and statistics

Breast cancer is a malignancy that arises in the cells of the mammary gland. This disease has become a major worldwide public health problem; current estimates indicate that breast cancer will affect approximately one in eight American women during their lifetime. According to American Cancer Society projections, there will be 192,370 estimated new cases of female breast cancer and 40,170 estimated deaths from this disease during 2009 in the USA alone (76). Breast cancer is the most commonly diagnosed cancer in women, and the second highest cause of cancer deaths in women. Breast cancer mortality rates among women in all age groups have been decreasing since 1991, and this trend is likely due to improvements in prevention, early detection and treatment. Current frontline treatments for breast cancer include surgery, radiation and drugs (chemotherapy and endocrine therapy). Finally, multiple risk factors have been defined for breast cancer, including reproductive events; increased age; lifetime exposure to endogenous and exogenous hormones (such as HRT and oral contraceptives); familial history; lifestyle risk factors such as obesity, alcohol, diet and physical activity; and genetic factors such as breast cancer susceptibility genes, and alcohol consumption (38).

1.3.2 The role of progestins and PR in breast cancer

1.3.2.1 Do progestins act as mitogens in the breast?

Many breast cancers are hormone responsive; in fact, approximately 75% of all breast tumors express estrogen receptor (ER), and more than half of these cancers also
express PR (109). The mitogenic effects of estrogen in breast cancer are well established, and antiestrogens such as tamoxifen are often used to block these actions in ER-positive breast cancers; however, the effects of progestins on breast cancer proliferation remain unclear. One of the most alarming clinical observations that associate progestin use with breast cancer risk came from the Women’s Health Initiative (WHI) trial, which assessed the risks and benefits of combination HRT (progestin + estrogen). The rationale behind combination HRT versus estrogen alone in healthy menopausal women is that progestins have been shown to block the proliferative and tumorigenic effects of estrogens in the uterus (32). Unfortunately, the WHI trial was stopped early due to an initial report of increased risk of invasive breast cancer in women receiving both hormones over women receiving estrogen alone, although later reports have put forth concerns regarding the statistical analysis and limitations of the study parameters (105, 154). The Scandinavian HABITS (hormonal replacement therapy after breast cancer – is it safe?) clinical trial was also terminated prematurely, due to findings of increased risk of recurrent breast cancer in women exposed to combination HRT (68). Finally, there is some evidence that use of combined oral contraceptives can increase breast cancer risk (1). Since the putative role of exogenous progestins as a breast cancer risk factor remains somewhat controversial, many investigators have focused on characterizing the effects of progestins on the in vitro proliferation of breast cancer cells.
1.3.2.2 PR and breast cancer cell cycle

In most breast cancer cell lines, PR activity is intrinsically linked to the estrogen receptor (ER) because it is necessary to treat with estrogens in order to induce sufficient PR expression. However, ER has been well established as a mitogen in breast cancer cells, so breast cancer cell lines that exhibit normal coupling of the ER and PR pathways are not useful for studies that focus on PR-specific actions in regulation of cell cycle. Thus, T47D breast cancer cells are often used as a model system because this cell line exhibits ER-independent, constitutive expression of both PR-A and PR-B (82, 195).

Treatment of T47D cells with a single dose of the synthetic progestin R5020 induces a biphasic proliferative response where cells undergo one round of proliferation before becoming quiescent and resistant to further PR-induced growth stimulation (125). The initial proliferative effects of progestins have been linked to PR-mediated induction of c-Myc and cyclin D1 transcription, which drives the transition from G$_1$ to S phase (123, 126, 211). Briefly, in a quiescent cell, hypophosphorylated Rb binds to the E2F transcription factor and blocks its activity (66). Upon growth stimulation, cyclin D1-cdk 4/6 complexes act to hyperphosphorylate Rb, thus releasing E2F and enabling it to induce transcription of genes needed for cell cycle progression from G$_1$ to S phase (98).

In contrast, the subsequent quiescent phase correlates with a decrease in cyclin D-cdk4/6 activity and subsequent dephosphorylation of Rb (59, 127). Furthermore, treatment with antiprogestins such as RU486 cause growth arrest of T47D cells in G$_1$ phase by inducing the cyclin-dependent kinase (cdk) inhibitors p21 and p27$^{Kip1}$ and
inhibiting cdk2 activity (59, 124). Importantly, although many molecular targets of PR have been well established, the overall mechanisms governing the opposing proliferative and growth inhibitory effects of PR remain unclear.

1.3.2.3 PR ligands in breast cancer treatment

The in vitro data on the proliferative effects of progestins on breast cancer cells has led some researchers to hypothesize that antiprogestins like mifepristone (RU486), which antagonize PR action, could be successful at blocking breast cancer progression. Initial studies of RU486 in animal models showed promising results, specifically that RU486 was as effective as tamoxifen at inhibiting breast tumor growth (8). In contrast, when RU486 was given to patients with metastatic, tamoxifen-resistant breast cancer in Phase I clinical trials, it only had a moderate response rate, although the PR status of these breast tumors was unknown (152).

Conversely, endocrine therapy with high doses of progestins has met with some clinical success in the treatment of advanced breast cancer (128). Several clinical trials have reported that a regimen of high-dose MPA or megestrol acetate (500-1500 mg/day) is as effective as tamoxifen in the treatment of advanced breast cancer in post-menopausal women (106). Furthermore, high-dose MPA has also proven useful as a second-line therapy in breast cancer patients that are resistant to tamoxifen or chemotherapy (34). Collectively, these studies indicate that hormonal therapy with high-dose progestins can serve as an effective front-line and second-line treatment for advanced, metastatic breast cancer.
While the molecular mechanisms by which PR regulates breast tumor growth and metastasis have not been fully elucidated, the aforementioned studies highlight the fact that PR has a complex role in breast cancer progression. It is therefore crucial that we obtain a better understanding of the effects of various PR ligands on downstream signaling pathways in the breast, because this knowledge will enable clinicians to make better-informed decisions regarding the use of PRMs in contraception, HRT and the treatment of hormone-responsive cancers.
2 Multimodal regulation of E2F1 gene expression by progestins

2.1 Introduction

2.1.1 Progesterone Receptor Signaling

The steroid hormone progesterone plays a central role in the development, growth and differentiation of the female reproductive system. The biological functions of progesterone are mediated by the two progesterone receptor isoforms, PR-A and PR-B, which belong to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors [for review, see (90)]. In the absence of ligand, PR is sequestered by heat shock proteins and maintained in an inactive state in the cytoplasm of target cells. Upon ligand binding, PR undergoes a conformational change that leads to its dissociation from the heat shock protein complex, an event that facilitates receptor dimerization and translocation into the nucleus. The receptor dimer is then capable of interacting with specific progesterone responsive elements (PREs) within target gene promoters. The DNA-bound receptor subsequently nucleates the assembly of large cofactor containing protein complexes that can either positively or negatively impact gene transcription.

In addition to this classical pathway of transcriptional activation, extra-nuclear PR can indirectly regulate gene expression by rapidly activating other signaling pathways. For instance, the N-terminal domain of PR contains a polyproline motif that has been shown to directly interact with the SH3 domains of c-Src and mediate rapid, non-genomic activation of c-Src family tyrosine kinases and the downstream mitogen-
activated protein kinase (MAPK) cascade (17). Additionally, progestins have been shown to rapidly activate the phosphoinositol 3-kinase (PI3K)/Akt/nuclear factor kappa B (NFκB) cascade and the Janus family of tyrosine kinases (JAK)/signal transducer and activator of transcription (STAT) signaling pathway in breast cancer cells (144, 157). Thus, through activation of these extranuclear signaling pathways, PR can regulate gene expression in a manner that is completely independent of its classic nuclear activities.

2.1.2 Cross talk between PR and other signaling cascades

While the nuclear and extranuclear actions of PR have been well studied in isolation, it is important to understand the mechanisms by which these pathways can interact and integrate to ultimately impact gene expression. Previous studies have established that the cross talk that occurs between PR and cytoplasmic signaling cascades is bidirectional and complex. On one hand, PR can activate rapid extranuclear signaling pathways such as that regulated by Src/MAPK and thereby modulate MAPK-dependent transcription; conversely, activated MAPKs can phosphorylate PR, or its attendant cofactors, and thereby modulate its ability to regulate target gene transcription (145). For example, MAPK kinase kinase 1 (MEKK1) has been shown to phosphorylate PR on Ser\textsuperscript{294}, which results in increased progestin-mediated transcription, as well as enhanced ligand-dependent receptor down-regulation (169).

Phosphorylation of cofactors can also have a dramatic impact on the transcriptional program set in motion by nuclear receptors. For instance, it was recently reported that phosphorylation of steroid receptor coactivator-3 (SRC-3; also known as
ACTR, AIB1, p/CIP, RAC3, or TRAM-1) can affect not only its activity, but different patterns of phosphorylation on SRC-3 can dictate the specificity of SRC-3 for various transcription factors (212). Although not yet studied in detail, it is likely, by extrapolation from studies of other NRs, that cofactor phosphorylation will also have a dramatic effect on PR transcriptional activity in cells. Cumulatively, studies highlighting the importance of the cross talk that occurs between the nuclear and extranuclear functions of PR have provided the impetus to define the molecular mechanisms by which these pathways are integrated and how disruption in these events can have pathological consequences.

2.2 Results

2.2.1 Inhibition of MAPK significantly alters gene regulation by progestins

In order to evaluate the degree to which the PR and MAPK signaling pathways converge at the level of gene transcription, we performed a microarray analysis to assess genome-wide changes in PR-dependent gene transcription in the presence of the MEK 1/2 inhibitor U0126 in T47D breast cancer cells. Gene expression profiling resulted in the identification of 2,510 probesets that were differentially expressed in response to treatment with R5020 for 24 h (Figure 2.1). These probesets mapped to 1,794 unique transcripts, of which 1,104 were up-regulated and 690 were down-regulated. Surprisingly, we observed that pre-treatment with U0126 altered progestin-mediated regulation of 1,395 genes.
Figure 2.1: Microarray analysis of T47D cells

Flow-chart schematic depicting breakdown of genes analyzed in T47D microarray.

To determine how many of these genes are potential direct PR target genes, we utilized Patser (65) to scan the 2 Kb upstream promoter regions with the PR position weight matrix (180) and found that 634 of the progestin-regulated genes have promoters that contain putative progesterone response elements (PREs) (Figure 2.29 in Discussion). Interestingly, an additional unbiased transcription factor enrichment analysis carried out using oPOSSUM (67) also detected a significant overrepresentation of E2F1 binding sites in the promoters of PR target genes; in fact, further analyses using Patser identified potential E2F1 binding sites in the promoters of 277 progestin-regulated genes (Figure 2.1). Furthermore, the microarray analysis showed that progestin treatment stimulated the transcription of classic E2F1 target genes such as CDC6, cyclin E and CDK2. These findings suggested that PR may indirectly impact transcription in cells by positively up-
regulating the expression and/or activity of E2F1, a key transcription factor involved in the regulation of the cell cycle.

### 2.2.2 Progestins induce expression of endogenous E2F1 mRNA and protein

Our hypothesis that PR could regulate the expression of E2F1 was supported by the microarray data, which indicated a 2.2-fold induction of E2F1 expression after treatment with R5020. To validate our microarray studies, we utilized qPCR to examine progestin-mediated regulation of endogenous E2F1 gene transcription in T47D:A18 cells. In order to reduce overall background levels of E2F, T47D cells were arrested in G₀ by serum starvation for 24 h. This cell cycle arrest was verified by propidium iodide cell cycle analysis (data not shown). In Figure 2.2, we demonstrate that synchronized T47D:A18 cells treated with R5020 for 18 h show an approximately 20-fold increase in E2F1 mRNA levels. While pre-treatment with U0126 did not affect regulation of the PR target gene S100P by R5020, inhibition of MAPK did reduce both progestin-mediated induction and basal expression of E2F1 mRNA levels. Western immunoblot analysis confirmed these results at the protein level; treatment with R5020 for 18 h dramatically increased E2F1 protein levels, and pre-treatment with U0126 partially blocked this effect (Figure 2.3).
Figure 2.2: Induction of endogenous E2F1 mRNA by R5020

Synchronized T47D:A18 cells were pre-treated with vehicle (veh) or 10 mM U0126 (U) for 30 min prior to addition of vehicle or 100 pM R5020 (R) for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).

Figure 2.3: Induction of endogenous E2F1 protein by R5020

Synchronized T47D:A18 cells were pre-treated with vehicle (veh) or 10 mM U0126 (U) for 30 min prior to addition of vehicle or 100 pM R5020 (R) for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for PR, E2F1 or ERK 1/2 as a loading control. A representative blot is shown.
Synchronized T47D:A18 cells were pre-treated with vehicle or 10 mM U0126 (U) for 30 min prior to addition of vehicle or 100 pM R5020 (R) for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. CDC2, CDC6, CDK2 or cyclin E1 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).

In addition, we confirmed that progestin treatment stimulates the transcription of classic E2F1 target genes such as CDC2, CDC6, cyclin E1 and CDK2 (Figure 2.4), suggesting that the E2F1 protein induced by PR is functional and active. We have not eliminated the possibility that PR may also exert direct effects on the expression of these genes. Importantly, we also observed a 12-fold increase in E2F1 mRNA levels after
treatment with R5020 in PR-positive BT483 breast cancer cells (Figure 2.5), indicating that the regulatory activities of PR on this target gene are not restricted to T47D cells.

**Figure 2.5: Induction of endogenous E2F1 mRNA by R5020 in BT483 cells**

Synchronized BT483 cells were treated with vehicle (veh) or 10 nM R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).

Finally, all of the experiments in this study were performed using concentrations of R5020 in the range of 100 pM to 10 nM, depending on the cell line and assay. In the course of the experiments, it was noted that in general, treatment of cells with 100 pM R5020 led to a greater induction of E2F1 mRNA and protein levels than higher doses such as 10 nM R5020 (data shown in Chapter 3). Because the focus of this study was to define the mechanisms underlying PR regulation of E2F1, the elucidation of the biphasic nature of E2F1 induction by R5020 will be addressed in Chapter 3.
2.2.3 PR-B is necessary for progestin-dependent regulation of E2F1 expression

To determine whether PR is necessary for R5020-mediated induction of E2F1 transcription, we examined the effects of progestin treatment on E2F1 expression in T47D:C42 cells (a PR-negative T47D subclone) that stably express a LacZ reporter gene (control cells), wild-type human PR-A, or PR-B (15). qPCR analysis demonstrated that R5020 does not induce E2F1 transcription in control cells or those expressing PR-A alone (Figure 2.6). However, induction of E2F1 expression was observed in cells in which wild-type PR-B was expressed (Figure 2.7).

![Figure 2.6: Progestin treatment does not induce E2F1 mRNA in cells expressing PR-A alone](chart)

The indicated T47D:C42 cells were synchronized and treated with vehicle (veh) or 10 nM R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).
Figure 2.7: PR-B mediates induction of E2F1 expression by R5020

The indicated T47D:C42 cells were synchronized and treated with vehicle (veh) or 10 nM R5020 for 16 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).

Given that R5020-mediated induction of E2F1 can be partially inhibited by U0126, we initially thought that the rapid, non-genomic actions of PR signaling through Src family kinases and the downstream MAPK pathway might be partly responsible for its regulation of E2F1. To further investigate this issue, we compared R5020 induction of E2F1 transcription in T47D:C42 cells that stably express wild-type PR-B or PR-BmPro, a mutant form of PR-B in which three key proline residues in the polyproline motif were replaced with alanines. This mutant PR receptor is unable to mediate rapid, non-genomic activation of Src family kinases or downstream MAPK, but its classical genomic functions remain intact (17). Interestingly, we determined that R5020 induces equal expression of E2F1 mRNA in cells expressing wild-type PR-B versus the mutant PR-BmPro version (Figure 2.7). From these data, we conclude that although MAPK activity
affects regulation of E2F1 expression, its activation is not dependent on direct PR signaling through Src family kinases.

Finally, treatment with R5020 has no effect on E2F1 mRNA levels in ER-/PR- human mammary epithelial cells (HMECs) infected with a control β-gal adenovirus, but infection with PR-B restores the ability of progestins to induce transcription of E2F1 in these cells (Figure 2.8). Collectively, these studies confirm that the PR-B isoform is both necessary and sufficient for progestin-mediated induction of E2F1 gene expression.

![Graph showing expression of PR-B in HMECs restores the ability of progestins to induce transcription of E2F1](image)

**Figure 2.8: Expression of PR-B in HMECs restores the ability of progestins to induce transcription of E2F1**

Human mammary epithelial cells (HMECs) were infected with a β-gal (negative control) or PR-B expressing adenovirus and subsequently treated with vehicle or 10 nM R5020 for 16 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).
2.2.4 Direct regulation of E2F1 transcription by PR

Next, we set out to define the mechanism by which PR regulates E2F1 expression. Given that R5020 is able to stimulate an increase in E2F1 mRNA levels as early as 4 h post-treatment (Figure 2.9), we suspected that the E2F1 gene might be a direct transcriptional target of PR. To investigate whether PR regulates E2F1 expression through the classic direct pathway of transcriptional regulation, we generated T47D:C42 cell lines that stably express wild-type PR-B or PR-B C587A, a zinc-finger mutant of PR-B that is unable to bind DNA (Figure 2.10). While R5020 treatment induced E2F1 expression in cells expressing wild-type PR-B, no significant change in E2F1 mRNA levels was evident in cells expressing the DNA-binding mutant of PR-B (Figure 2.11). Therefore, we conclude that the DNA-binding capacity of PR is required for progestin regulation of E2F1.

![Bar chart of S100P and E2F1 expression levels](image)

**Figure 2.9: Early timecourse of E2F1 induction by R5020**

Synchronized T47D:A18 cells were treated with vehicle (veh) or 100 pM R5020 for the indicated time points. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).
Figure 2.10: Expression of PR-B in T47D:C42 stable cell lines

Synchronized T47D:C42 cells were treated with vehicle (V) or 10 nM R5020 (R) for 24 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for PR or GAPDH as a loading control. This Western blot control confirms that T47D:C42-hPR-B and T47D:C42-hPR-B-C587A stable cells express similar levels of PR-B. A representative blot is shown.

Figure 2.11: The DNA-binding capacity of PR-B is required for progestin regulation of E2F1

The indicated T47D:C42 cells were synchronized and treated with vehicle or 10 nM R5020 for 24 h. Cells were lysed and RNA was isolated and reverse transcribed. FKBP51 or 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).

We were unable to identify any putative progesterone response elements (PREs) within the promoter sequence surrounding E2F1 using Transcription Element Search.
Software (TESS) (164). Furthermore, ChIP-chip analysis of T47D cells treated with progesterone did not identify any PR-binding sites within the 2 Kb upstream promoter region of the *E2F1* gene (Chromosome 20:31,737,871-31,739,871; our unpublished data). However, a genome-wide ChIP-chip analysis did reveal that progesterone-activated PR is recruited to two proximal enhancer sites, located ~2.3 Kb downstream of E2F1 (Figure 2.12). We noted that sites #1 and #2 are located within the *XB51* locus; however, although R5020 treatment led to a 20-30 fold induction of E2F1 mRNA, XB51 was consistently induced less than 2-fold (data not shown).

**Figure 2.12: Location of potential PR binding sites surrounding E2F1**

Schematic depicting the locations of 2 proximal enhancer elements and 4 distal enhancer elements located around E2F1 that were identified in ChIP-chip experiments as potential PR binding sites.

Next, we performed ChIP studies to test whether R5020-activated PR is recruited to these proximal enhancer elements. Recruitment of PR to a previously characterized intronic PRE within *FKBP51* was used as a positive control for PR binding (100). Our ChIP analysis confirmed that ligand-bound PR associates with site #1, with a 5-fold increase in recruitment at 1-2 h post-treatment with R5020 (Figure 2.13). Moreover, PR remains associated with site #1 as late as 18 h post-treatment. Unfortunately, we were
unable to ascertain whether PR binds to site #2 due to poor PCR efficiency despite attempts with multiple sets of PCR primers.

![Graph showing recruitment of PR to proximal enhancer elements nearby E2F1](image)

**Figure 2.13: Recruitment of PR to proximal enhancer elements nearby E2F1**

Synchronized T47D:A18 cells were treated with vehicle or 10 nM R5020 for the indicated time points. Cells were harvested after cross-linking and subjected to immunoprecipitation with either mouse IgG control (mlG) or PR antibody. Following reversal of cross-linking, DNA was isolated and subjected to qPCR analysis using primers spanning a region in FKBP51 (positive control), stromelysin (negative control), or the potential PR-binding region proximal to E2F1 (Proximal Site #1). The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment.

In addition to the proximal enhancer elements, the ChIP-chip data also identified 4 distal enhancer sites located ~29.5 Kb upstream of E2F1 (Figure 2.12). Our subsequent
ChIP studies confirmed significant recruitment of PR to all four distal sites in a ligand-dependent manner (Figure 2.14). Sites #5 and #6 are located within intronic regions of ZNF341, a gene that is weakly regulated by PR; sites #3 and #4 are respectively located within intronic and promoter regions of PXMP4, a gene that is positively regulated by R5020 treatment (data not shown). Studies are currently ongoing to determine whether recruitment of PR to these distal sites is involved in progestin regulation of E2F1; however, TESS analysis did confirm that all six sites contain putative PREs. Thus, we have identified both proximal and distal enhancer elements to which PR could bind and directly regulate expression of E2F1.
Figure 2.14: Recruitment of PR to distal enhancer elements nearby E2F1

Synchronized T47D:A18 cells were treated with vehicle or 10 nM R5020 for the indicated time points. 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 the four potential PR-binding regions distal to E2F1 (Distal Sites #3–#6). The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment.

To further verify that E2F1 is a direct target of PR action, we pre-treated T47D:A18 cells with or without the translational inhibitor cycloheximide, followed by addition of vehicle or R5020 for 18 h. Using qPCR, we determined that cycloheximide did not inhibit induction of SGK1 (serum and glucocorticoid regulated kinase), an established primary target of PR (15). In contrast, we observed that pre-treatment with
cycloheximide partially inhibits R5020-mediated induction of E2F1 transcription (Figure 2.15), signifying that nascent protein synthesis is required to achieve maximal PR induction of E2F1 expression. Furthermore, while R5020 can up-regulate E2F1 mRNA levels by early timepoints such as 4-6 h post-treatment, maximal induction of E2F1 transcription by R5020 is not achieved until 18 h post-treatment (data not shown). These data prompted us to consider that the ligand-dependent actions of PR on the E2F1 gene may involve additional indirect regulatory pathways.

**Figure 2.15: Cycloheximide partially inhibits R5020-mediated induction of E2F1 transcription**

Synchronized T47D:A18 cells were pre-treated with vehicle or 5 or 10 mg/mL cycloheximide (CHX) for 1 h prior to addition of vehicle (veh) or 100 pM R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. SGK1 or 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).
2.2.5 R5020 treatment increases phosphorylation of Rb and recruitment of E2F1 to its own promoter

It is well known that E2F1 can up-regulate its own expression by binding to previously defined E2F binding sites within its own promoter (78). Therefore, we hypothesized that E2F1 protein produced as a result of direct PR regulation could act to further amplify progestin-induced E2F1 transcription by activating a positive feedback loop. Since the ability of E2F family members to influence transcription of target genes is regulated by the phosphorylation status of the retinoblastoma protein Rb, we first examined the effects of progestin treatment on the phosphorylation of Rb. While a cascade of phosphorylation events regulates Rb activity, we chose to focus on the phosphorylation of three sites in particular. Prior studies indicate that sequential phosphorylation of Rb on Ser$^{780}$, followed by Ser$^{807/811}$, is important for release of E2F from Rb and optimal activation of downstream E2F target gene transcription, respectively (Figure 2.16) (98).

![Figure 2.16: E2F1 can stimulate its own transcription through a positive feedback loop](image)

Schematic depicting hyperphosphorylation of Rb and subsequent release of E2F, which allows E2F to bind its own promoter and increase transcription in a positive feedback loop.
Western blot analysis with total and phospho-specific Rb antibodies shows that treatment with progestins for 9 to 18 h led to an increase in phosphorylation of Rb at Ser$^{780}$ and Ser$^{807/811}$, as well as an overall increase in total levels of hyperphosphorylated Rb (Figure 2.17). However, we saw no increase in phosphorylation of Rb at Ser$^{780}$ and Ser$^{807/811}$ or change in total levels of hyperphosphorylated Rb at any of the earlier timepoints that we examined (data not shown). Furthermore, we discovered that this progestin-mediated increase in Rb phosphorylation can be partially inhibited by pretreatment with U0126, and this corresponds with a reduction in the amount of E2F1 protein induced by an 18 h treatment with R5020 (Figure 2.18).

**Figure 2.17: Induction of Rb hyperphosphorylation by progestins**

Synchronized T47D:A18 cells were treated with vehicle (veh) or 100 pM R5020 for the indicated time periods. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total Rb, Rb phosphorylated on Ser780 (p-Rb Ser 780), Rb phosphorylated on Ser807/811 (p-Rb Ser 807/811), E2F1 or GAPDH as a loading control. ns = non-specific band. A representative blot is shown.
Figure 2.18: MAPK inhibition decreases induction of Rb hyperphosphorylation by progestins

Synchronized T47D:A18 cells were pre-treated with vehicle (veh) or 10 mM U0126 (U) for 30 min prior to addition of vehicle or 100 pM R5020 (R) for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total Rb, Rb phosphorylated on Ser780 (p-Rb Ser 780), Rb phosphorylated on Ser807/811 (p-Rb Ser 807/811), E2F1 or ERK 1/2 as a loading control. ns = non-specific band. A representative blot is shown.

Since we observed an increase in Rb phosphorylation at 9-18 h post-treatment with R5020, we hypothesized that any progestin-mediated increase in recruitment of E2F1 to its own promoter might correspondingly occur within this timeframe. To address this question, we performed ChIP experiments in T47D:A18 cells to measure E2F1 occupancy at its own promoter. As expected, treatment with R5020 for 1-2 h did not result in a significant increase in E2F1 recruitment to the region of the E2F1 promoter containing E2F binding sites (Figure 2.19). In contrast, while ligand-bound PR is already recruited to enhancer elements near the E2F1 gene at these early timepoints (Figures 2.13 and 2.14), Rb remains hypophosphorylated and bound to E2F1, thereby preventing it
from binding to the promoters of target genes. However, by 18 h post-treatment, Rb has become hyperphosphorylated, which frees E2F1 and enables it to interact with its cognate response element in the E2F1 promoter. Correspondingly, ChIP studies showed a significant progestin-mediated increase in recruitment of E2F1 to its own promoter at this later timepoint (Figure 2.19). Collectively, these data indicate that PR acts indirectly to further amplify expression of E2F1 by stimulating phosphorylation of Rb and recruitment of E2F1 to its own promoter. Inhibition of MAPK decreases the ability of PR to stimulate hyperphosphorylation of Rb; this is one mechanism by which U0126 can act to impair progestin-mediated induction of E2F1 expression.

Figure 2.19: R5020 further amplifies E2F1 transcription by activating a positive feedback loop

Synchronized T47D:A18 cells were treated with vehicle or 100 pM R5020 for the indicated time points. Cells were harvested after cross-linking and subjected to immunoprecipitation with either mouse IgG control (mIgG) or E2F1 antibody. Following reversal of cross-linking, DNA was isolated and subjected to qPCR analysis using primers spanning a region in the E2F1 promoter containing E2F binding sites. The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment.
2.2.6 GC-rich DNA within the E2F1 promoter is important for progestin-mediated induction of E2F1 expression

During our search for an indirect pathway through which PR could modulate E2F1 expression, we searched for additional regulatory elements located within the E2F1 promoter that might be involved in this response. In addition to the previously mentioned E2F binding sites, the E2F1 promoter also contains many GC-rich regions of DNA, which commonly serve as binding sites for members of the Specificity Protein/Krüppel-like Factor (Sp/KLF) transcription factor superfamily (94). Previous studies have suggested that a member of the Sp/KLF superfamily may play a role in the regulation of the E2F1 promoter; more specifically, loss of a small 82-bp region (-204 to -122 in Figure 2.20) that contains several clusters of GC-rich DNA results in reduced activity of the E2F1 promoter (78). Therefore, we were intrigued by the observation that a number of Sp/KLF family members were induced by R5020 in our array; furthermore, oPOSSUM identified an enrichment of Sp1 sites in the promoters of PR-regulated genes.

**Figure 2.20: The E2F1 promoter**

Schematic depicting regulatory elements within the E2F1 promoter. The indicated regions of GC-rich DNA can serve as binding sites for members of the Sp/KLF superfamily.

To determine whether binding of an Sp/KLF family member to GC-rich DNA within the E2F1 promoter is important for progestin-dependent E2F1 induction, we pre-
treated T47D:A18 cells with Mithramycin A, an antibiotic that binds to GC-rich DNA and blocks recruitment of transcription factors to these regions (117). Pre-treatment with Mithramycin A suppresses R5020-mediated induction of E2F1 transcription, but does not decrease progestin-induced mRNA levels of the primary PR target gene SGK1, although basal levels of SGK1 mRNA did increase (Figure 2.21). Thus, we hypothesized that a transcription factor belonging to the Sp/KLF superfamily may be involved in PR-mediated induction of E2F1 expression.

Figure 2.21: Effects of Mithramycin A on progestin induction of E2F1 expression

Synchronized T47D:A18 cells were pre-treated with vehicle (veh) or 200 nM Mithramycin A (MitA) for 30 min, then treated with vehicle or 100 pM R5020 for 18 h. Cells were lysed and RNA was isolated and reverse transcribed. SGK1 or E2F15 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 Krüppel-like factor 15 (KLF15) is required for maximal induction of E2F1 expression by PR

To further interrogate the potential involvement of an Sp/KLF family member in progestin regulation of E2F1 transcription, we utilized qPCR analysis to examine the expression of various Sp/KLF family members in synchronized T47D:A18 cells treated with 100 pM R5020 for 18 h. In fact, R5020 induces transcription of several Sp/KLF family members, including Sp1, KLF4, KLF9, and KLF15 (Figure 2.22).

![Figure 2.22: Induction of Sp/KLF family members by R5020](image)

Synchronized T47D:A18 cells were treated with vehicle (veh) or 100 pM R5020 for 18 h. Cells were lysed and RNA was isolated and reverse transcribed. Sp1, KLF4, KLF9 or KLF15 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).

KLF15 was the most robustly induced Sp/KLF family member among those that we examined; furthermore, R5020 increased KLF15 mRNA levels rapidly within 2 h (Figure 2.23), which preceded PR-mediated induction of E2F1 expression (Figure 2.9). Additionally, qPCR studies with cycloheximide confirm that KLF15 is a direct PR target.
(Figure 2.24). Therefore, we chose to evaluate the potential role of KLF15 in PR-mediated induction of E2F1 expression.

**Figure 2.23: Early timecourse of KLF15 induction by R5020**

Synchronized T47D:A18 cells were treated with vehicle (veh) or 100 pM R5020 for the indicated time points. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or KLF15 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).

**Figure 2.24: Effects of cycloheximide treatment on R5020-mediated induction of KLF15 transcription**

Synchronized T47D:A18 cells were pre-treated with vehicle or 5 or 10 mg/mL cycloheximide (CHX) for 1 h prior to addition of vehicle (veh) or 100 pM R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. SGK1 or KLF15 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).
Using a position weight matrix previously described for KLF15 (135), the E2F1 promoter was scanned for putative KLF15 binding motifs using TESS. This analysis identified 3 putative KLF15 binding sites within the 82-bp GC-rich DNA region mentioned previously (Figure 2.25). Unfortunately, KLF15 antibodies suitable for ChIP analysis are not yet available, and thus we could not directly examine whether KLF15 is recruited to these putative binding sites in the E2F1 promoter. As an alternative approach to probe the involvement of KLF15 in E2F1 gene regulation, we utilized luciferase assays to explore the connection between KLF15 and the E2F1 promoter. T47D:A18 cells were transiently transfected with a series of reporter gene constructs that contain successively smaller regions of the E2F1 promoter, in combination with increasing amounts of wild-type KLF15 or a KLF15 mutant that lacks the N-terminal DNA binding domain (KLF15 NΔ291). Wild-type KLF15 increased activation of the longer E2F1 promoter fragments in a dose-dependent manner, but was unable to activate the smallest promoter fragment (-122), which lacks the GC-rich DNA region containing the putative KLF15 binding sites (Figure 2.26). In contrast, addition of the mutant KLF15 NΔ291 construct did not affect activation of any E2F1 reporter constructs, indicating that the DNA-binding ability of KLF15 is required for induction of E2F1 activity.
Figure 2.25: Potential KLF15 binding sites within the E2F1 promoter

Schematic depicting a position weight matrix (PWM) for KLF15, which was created based on previously characterized KLF binding sites. Transcription Element Search Software (TESS) was utilized to identify several putative KLF15 binding sites within the 82-bp GC-rich region of the E2F1 promoter that is missing in the -122 luciferase construct. The above diagram depicts the location of the three strongest matches.
Figure 2.26: The DNA-binding capacity of KLF15 is required for activation of E2F1 transcription

T47D:A18 cells were transiently co-transfected with various hE2F1-luc promoter fragment reporters along with increasing amounts of a vector expressing wild-type KLF15 or the KLF15 NΔ291 deletion mutant for 48 h, then harvested and assayed for luciferase activity. Luciferase values were normalized to β-galactosidase control. Data are the mean relative light units (RLUs) ± SEM for one representative experiment performed in triplicate. Inset, Western blot control confirming equal expression of His-tagged KLF15 variants using GAPDH as a loading control.

To further implicate KLF15 in progestin regulation of E2F1 expression, we performed knockdown studies using two independent siRNAs targeting KLF15. Since we could not identify a reliable, working antibody that would detect KLF15 expression in T47D:A18 cells, we were unable to confirm knockdown of KLF15 at the protein level. However, qPCR analysis demonstrates that both siRNAs can inhibit basal and R5020-mediated induction of KLF15 mRNA levels to various extents, and even partial knockdown of KLF15 transcription had an inhibitory effect on R5020-mediated induction of E2F1 mRNA levels (Figure 2.27). In contrast, knockdown of KLF15 did not decrease the regulation of other classic PR target genes such as FKBP51. Taken together, these
findings indicate that progestin-mediated induction of KLF15 is required for maximal induction of E2F1 expression by PR.

Figure 2.27: Knockdown of KLF15 reduces R5020-mediated induction of E2F1 expression

T47D:A18 cells were transiently transfected with Stealth siRNAs targeting KLF15 (siKLF15 2-3) or a negative control siLuciferase (siLuc) at a final concentration of 100 nM for 48 h. Cells were synchronized by serum-starvation for 24 h and then treated with vehicle (veh) or 100 pM R5020 (R) for 18 h. FKBP51, KLF15 or 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

We show that PR is a component of several distinct pathways that function both directly and indirectly to positively up-regulate E2F1 expression in breast cancer cells (Figure 2.28). Firstly, PR directly regulates E2F1 transcription by binding to proximal and distal enhancer sites located near E2F1. Secondly, progestins induce the hyperphosphorylation of Rb, which results in increased recruitment of E2F1 to its own promoter, thereby activating a positive feedback loop that further amplifies its transcription. Finally, PR induces expression of KLF15 and potentially other Sp/KLF
family members, which bind to GC-rich regulatory regions within the E2F1 promoter and further activate transcription. Together, these pathways represent a complex multimodal regulatory system in which the combined actions of each component are required for maximal progestin-mediated up-regulation of E2F1 transcription.

Figure 2.28: Model of multimodal regulation of E2F1 by progestins

Ligand-bound PR can bind to proximal and distal enhancer sites located near E2F1 and directly regulate E2F1 transcription. PR can also act indirectly through hyperphosphorylation of Rb and induction of KLF15 expression to achieve further progestin-mediated regulation of E2F1 expression in T47D cells.

2.3.1 T47D cell line as a model system

In most breast cancer cell lines, estrogens are important for regulation of PR expression; however, the estrogen receptor (ER) has previously been shown to induce
expression of E2F1, and we wanted to concentrate solely on PR-specific regulation of E2F1 expression. Therefore, we chose T47D cells as a model system for our studies because in this cell line, PR expression is uncoupled from ER signaling (82, 195).

2.3.2 Biological significance of E2F action in the breast

Given that progestins can stimulate proliferation of T47D cells in vitro and when propagated as xenografts in vivo, it was not unexpected to see that PR also modulates expression of E2F1, a transcription factor that controls cell cycle progression. However, we noted that E2F1 expression was also induced in response to progestins in BT483 breast cancer cells (Figure 2.5), and in ER-negative/PR-negative human mammary epithelial cells (HMECs) infected with a PR-B adenovirus (Figure 2.8); model systems where progestins do not stimulate proliferation. Importantly, the downstream biological effects of E2F1 are not limited to regulation of cell proliferation; indeed, E2F1 has been implicated in other critical processes such as DNA damage response, checkpoint control, and apoptosis (35). Defining the role(s) of these additional processes in PR biology is an area of continued exploration in our group. Additionally, the microarray analysis showed that treatment of T47D cells with R5020 stimulated the expression of E2F2 and E2F7; further studies are necessary to explore the effects of these E2F family members on PR signaling.

2.3.3 Cross talk between PR and the MAPK cascade

The initial purpose of our microarray study was to determine the overall involvement of the MAPK signaling pathway in PR regulation of target gene
transcription. We were surprised to find that the expression levels of almost 80% of the 1,794 PR target genes identified in this analysis were affected by pre-treatment with the MEK 1/2 inhibitor U0126 (Figure 2.1). Of course, since inhibition of MAPK reduces progestin-mediated up-regulation of E2F1 expression (Figures 2.2 and 2.3), any PR target genes that are co-regulated by this protein would be correspondingly affected. One explanation for the inhibitory effect of U0126 on progestin-mediated induction of E2F1 expression is the observation that MAPK inhibition partially suppressed PR-mediated hyperphosphorylation of Rb (Figure 2.18), which is necessary for release of E2F and activation of the positive feedback loop (Figure 2.16).

While the mechanism(s) by which progestins induce hyperphosphorylation of Rb have not been fully elucidated, it has been established that treatment of T47D cells with progestin leads to induction of cyclins D1 and E and increased activity of the cyclin D1/cdk4 complex (171, 182, 190), which has been implicated in phosphorylation of several sites on Rb (208). Previous studies have reported that progestin induction of cyclin D1 is dependent on rapid PR activation of the Src/MAPK pathway (15); therefore, we initially hypothesized that direct interactions between PR and Src family kinases might activate MAPK and contribute to progestin regulation of E2F1. However, we determined that R5020 effectively induces expression of E2F1 mRNA in cells expressing either wild-type PR-B or the mutant PR-BmPro (Figure 2.7), which cannot directly interact with c-Src or mediate rapid, non-genomic activation of Src/MAPK signaling.
However, other studies have proposed an alternative mechanism for rapid activation of MAPK signaling by progestins, whereby PR interacts with unliganded ER, which in turn activates the Src/MAPK signaling pathway (10, 116). Furthermore, a recent study reported that progestin induction of cyclin D1 requires both the DNA binding domains of PR, which allow PR to bind directly to distal regions of the cyclin D1 promoter, and the two ER-interacting domains (ERID) of PR, which allow PR to interact with ER to achieve rapid activation of Src/MAPK (147). Additional studies are necessary to determine whether PR activation of MAPK through this alternative, ER-dependent pathway and subsequent induction of cyclin D1 is the mechanism leading to progestin-mediated hyperphosphorylation of Rb, and subsequent induction of the positive feedback loop that amplifies E2F1 expression. Interestingly, we noted that the magnitude of PR-mediated induction of E2F1 expression in ER-negative cell lines, such as T47D:C42 cells (Figure 2.7) or HMECs (Figure 2.8), was not as great as that achieved by progestins in ER-positive cell lines, such as T47D:A18 cells (Figure 2.2) or BT483 cells (Figure 2.5). The significance of this observation is currently under investigation.
2.3.4 Co-regulation of PR target gene expression

Figure 2.29: Overlap of potential transcription factor binding site occurrence within the promoters of progestin-regulated genes

Patser was utilized to scan the 2 Kb upstream promoter regions of progestin-regulated genes with PWMs for PR, E2F1 and SP1, using a p-value cutoff of $1 \times 10^{-4}$ and a background model sampled from the set of all progestin-regulated promoters. A previously defined PWM for PR was utilized, and the PWMs for E2F1 and Sp1 were obtained from JASPAR. This Venn diagram depicts the overlap in occurrence of putative binding sites for these transcription factors.

Bioinformatic analyses revealed a 277-gene subset of progestin-regulated transcripts that was enriched for E2F binding sites (Figure 2.1); this subset includes classic E2F1 target genes such as CDC6, cyclin E and CDK2. However, it is currently unclear whether the effects of progestins on these genes and others are mediated solely by
secondary E2F1 actions, or whether PR also directly regulates their transcriptional activity. Analyses with Patser showed that 99 progestin-regulated genes contain both putative PREs and E2F1 binding sites within their promoters (Figure 2.29), and this may indicate a trend of co-regulation of target genes by direct actions of PR and E2F1. Interestingly, since the expression of as many as 277 R5020-regulated genes may be modulated by E2F1, a target of PR-B but not PR-A (Figures 2.6 and 2.7), it is possible that regulation of E2F1 by the PR-B isoform could be an important factor that contributes to the vastly different profiles of PR-A and PR-B as transcriptional regulators.

Similarly, several pieces of data suggest a trend of co-regulation of target genes by PR and members of the Sp/KLF superfamily. For instance, pre-treatment with Mithramycin A affected R5020-mediated induction of many downstream PR target genes that we examined; moreover, we observed that knockdown of KLF15 inhibited R5020 induction of several PR target genes (data not shown). Bioinformatic analyses using Patser revealed that out of the 1,794 PR target genes detected in our microarray study, the promoters of 1,372 genes contain putative GC-rich binding sites for Sp/KLF family members (Figure 2.29). Studies are currently ongoing to determine whether cooperation between PR and KLF15 and/or other SP/KLF family members in the regulation of gene transcription constitutes a more global model of PR function.

While the extent to which PR engages in multimodal regulation of target genes remains to be determined, the data we have generated in this study indicates that the ability of PR to induce the expression of E2F and Sp/KLF family members and their
resulting impact on gene expression provides a mechanism to explain secondary, cycloheximide-sensitive responses to progestins. In general, the indirect secondary responses that are stimulated by progestins have been less studied than primary transcriptional responses; however, this area of PR signaling deserves more attention, since the regulation of target gene expression by PR-stimulated transcription factors can dramatically influence the overall transcriptional program set into motion by progestins. In the context of PR regulation of E2F1 transcription, secondary factors such as E2F1 and KLF15 act to reinforce progestin-mediated induction of E2F1 expression, but E2F and Sp/KLF family members may act to suppress PR actions on other target genes.

2.3.5 Progestin up-regulation of KLF15

Finally, induction of KLF15 expression by PR has ramifications that extend beyond its role in progestin-mediated regulation of E2F1. KLF15 is a recently discovered transcription factor, and the transcriptional mechanisms that regulate KLF15 promoter activity are poorly understood; however, several recent studies support a role for NRs in regulation of KLF15 expression. In ovariectomized mice, treatment with estradiol and progesterone up-regulates KLF15 expression in the uterine epithelium (138). In addition, dexamethasone treatment induces KLF15 expression in chondrocytes (75), and both corticosterone and the glucocorticoid receptor-specific agonist, cortivazol, up-regulate KLF15 expression in cardiomyocytes (214). Furthermore, little is known about the biological function(s) of KLF15 in the breast. In our qPCR analysis of breast cancer cells, we observed that basal transcription of KLF15 was low; in contrast, KLF15 is highly
expressed in the liver, kidney, heart, and skeletal muscle (193). Studies involving KLF15 in other tissues have revealed an emerging role for KLF15 in regulation of metabolic processes such as glucose homeostasis (57) and lipid accumulation (37). It is clear that further studies are warranted to determine how progestin-mediated activation of KLF15 signaling may impact metabolic signaling processes in the breast.

2.3.6 Multimodal gene regulation by PR

In conclusion, although E2F1 transcription is impacted by the direct interaction of PR with the regulatory regions near \( E2F1 \), we also established that maximal induction of E2F1 expression by progestins requires the actions of additional transcription factors, such as E2F1 and KLF15, on the E2F1 promoter. The same may be true for a much larger subset of PR target genes. In fact, we suspect that PR often acts in concert with these and other secondary factors to co-regulate target gene expression, depending on cell- or tissue-specific context. These results suggest a paradigm for multimodal PR gene regulation that entails cooperation between direct and indirect pathways of PR signaling to achieve the desired downstream transcriptional cascade.
3 Low-dose versus high-dose progestins: less is more?

3.1 Introduction

Progesterone is a naturally occurring steroid hormone that 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. In the breast and other tissues of the female reproductive system, progesterone plays an important role in normal development and function. Therefore, many synthetic PR modulators (PRMs) have been developed over the past few decades to manipulate the downstream biology of PR, and are widely prescribed for purposes including contraception, termination of pregnancy, and hormone replacement therapy (HRT).

However, progestins and PR have also been implicated in disease pathologies such as breast cancer. Many breast cancers are hormone responsive; in fact, approximately 75% of all breast tumors express estrogen receptor (ER), and more than half of these cancers also express PR (109). While the mitogenic effects of estrogen in breast cancer are well established, and antiestrogens such as tamoxifen are often used to block these actions in ER-positive breast cancers, the effects of progestins on breast cancer proliferation remain unclear. One of the most alarming clinical observations that associate progestin use with breast cancer risk came from the Women’s Health Initiative (WHI) trial, which assessed the risks and benefits of combination HRT. Progestins are often used in combination with estrogens in HRT for healthy menopausal women due to the ability of progestin to block the proliferative and tumorigenic effects of estrogens in
the uterus (32). In the WHI trial, post-menopausal women received a combination of conjugated equine estrogen (CEE, 0.625 mg/day) and low-dose medroxyprogesterone acetate (MPA, 2.5 mg/day) (154). Unfortunately, the WHI trial had to be stopped early due to an increased risk of invasive breast cancer in women receiving both hormones over women receiving estrogen alone. Furthermore, progestins have been shown to have proliferative effects in various breast cancer cell lines in vitro and in animal models of mammary tumorigenesis (26). Therefore, the current treatment paradigm is to utilize the lowest effective dose of progestins in both contraceptives and HRT, in hopes of maximizing the benefits while reducing the risk of serious side effects.

On the other hand, endocrine therapy with high doses of progestins has met with some clinical success in the treatment of advanced breast cancer (128). Several clinical trials have reported that a regimen of high-dose MPA or megesterol acetate (500-1500 mg/day) is as effective as tamoxifen in the treatment of advanced breast cancer in post-menopausal women (106). Furthermore, high-dose MPA has also proven useful as a second-line therapy in breast cancer patients that are resistant to tamoxifen or chemotherapy (34). Collectively, these studies indicate that hormonal therapy with high-dose progestins can serve as an effective front-line and second-line treatment for advanced, metastatic breast cancer.

While the molecular mechanisms by which PR regulates breast tumor growth and metastasis have not been fully elucidated, the aforementioned studies highlight the fact that PR has a complex role in breast cancer progression. It is crucial that we obtain a
better understanding of the effects of low-dose versus high-dose progestins on PR signaling pathways in the breast, because this knowledge will enable clinicians to make better-informed decisions regarding the use of PRMs in contraception, HRT and the treatment of hormone-responsive cancers. Therefore, we undertook the present study to investigate the consequences of treatment with low-dose versus high-dose progestins on downstream PR biology in breast cancer cells.

3.2 Results

3.2.1 The impact of low-dose versus high-dose progestins on downstream PR biology

3.2.1.1 Activation of target gene expression

3.2.1.1.1 Induction of E2F1 transcription by R5020

To investigate the effects of low-dose versus high-dose progestins on the downstream activities of PR, we began by examining the expression of several PR target genes in T47D:A18 breast cancer cells treated with varying concentrations of the synthetic progestin R5020. Treatment with increasing amounts of R5020 stimulates a dose-dependent increase of the mRNA levels of the classic PR target gene S100P, displaying a traditional sigmoid dose response curve in which the maximum induction reaches a plateau at high doses of R5020 between 1 to 10 nM (Figure 3.1).

Therefore, it is not surprising to find in a survey of the literature that most studies involving PR are conducted using high doses of progestins within the range of 1 to 100 nM, due to the assumption that the maximal effects of PR on all target genes will be
manifest when the receptor is saturated with ligand. However, we were surprised to discover that treatment of T47D:A18 cells with increasing amounts of R5020 had a biphasic effect on the transcriptional activation of another PR target gene, E2F1. In contrast to the dose response curve for S100P, low-dose 100 pM R5020 stimulated maximal induction of E2F1 mRNA levels, and this induction was greatly reduced after treatment with higher doses of R5020 such as 1 or 10 nM (Figure 3.1).

![Figure 3.1: Biphasic regulation of E2F1 transcription by R5020](image)

Synchronized T47D:A18 cells were treated with increasing concentrations of R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).

3.2.1.1.2 Transcriptional activation of other PR target genes by R5020

To ensure that the biphasic dose response curve of E2F1 induction elicited by R5020 was not an isolated phenomenon restricted to a single PR target gene, we obtained dose response curves for a panel of PR target genes in T47D:A18 cells treated with increasing amounts of R5020. Several of the PR target genes exhibited traditional
sigmoid dose response curves, with maximal induction of gene transcription occurring after treatment with high-dose 10 nM R5020. However, biphasic dose response curves were observed for a substantial subset of the PR target genes that we examined, with maximal induction of gene transcription occurring at low-dose 100 pM R5020 treatment.

A summary of these results is shown in Table 3.1.

**Table 3.1: Classification of PR target genes by their response to low-dose versus high-dose R5020 treatment**

<table>
<thead>
<tr>
<th>PR Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class I: Classical sigmoid R5020 dose response curve</strong></td>
</tr>
<tr>
<td>S100P</td>
</tr>
<tr>
<td>KLF4</td>
</tr>
<tr>
<td>KLF9</td>
</tr>
<tr>
<td><strong>Class II: Biphasic R5020 dose response curve</strong></td>
</tr>
<tr>
<td>SGK1</td>
</tr>
<tr>
<td>E2F1</td>
</tr>
<tr>
<td>E2F2</td>
</tr>
<tr>
<td>DP-1</td>
</tr>
<tr>
<td>Cyclin D1</td>
</tr>
<tr>
<td>Sp1</td>
</tr>
<tr>
<td>KLF15</td>
</tr>
</tbody>
</table>

### 3.2.1.1.3 Induction of E2F1 mRNA levels by progesterone

To determine whether this biphasic regulation of a subset of PR target genes is a phenomenon caused exclusively by the synthetic PR ligand R5020, we examined the effects of the naturally-occurring PR ligand progesterone on E2F1 expression in T47D:A18 cells. Interestingly, we observed similar patterns in the induction of S100P and E2F1 transcription after treatment with progesterone compared to R5020. Increasing amounts of progesterone stimulated increasing levels of S100P mRNA, with maximal
PR-mediated S100P transcription occurring after treatment with high-dose 10 nM progesterone (Figure 3.2). In contrast, treatment of T47D:A18 cells with increasing amounts of progesterone had a biphasic effect on the transcriptional activation of E2F1, although the dose response curve was shifted slightly to the right and maximal induction of E2F1 transcription occurred at 1 nM progesterone (Figure 3.2). One possible explanation for this shift could be that progesterone has a lower affinity for PR than R5020 (140).

![Graphs showing regulation of S100P and E2F1 by progesterone](image)

**Figure 3.2: Biphasic regulation of E2F1 transcription by progesterone**

Synchronized T47D:A18 cells were treated with increasing concentrations of progesterone for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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).

### 3.2.1.1.4 Comparison of E2F1 induction by low-dose progestins versus low-dose estrogens or androgens

Given that the estrogen receptor (ER) and androgen receptor (AR) have previously been shown to induce E2F1 expression and/or activity (102, 179), we next set out to determine whether these nuclear receptors also regulate E2F1 transcription in a
biphasic manner. Preliminary data showed that treatment of T47D:A18 cells with increasing amounts of the AR ligand dihydrotestosterone (DHT) did not have a biphasic effect on E2F1 transcription; maximal induction of E2F1 mRNA levels occurred at 10 nM DHT, the highest dose that we tested (Figure 3.3). Similarly, the dose response curve obtained after treating T47D:A18 cells with increasing amounts of the ER ligand estradiol (E2) did not appear to be significantly biphasic (Figure 3.3). While there may have been a slight drop in E2F1 mRNA levels after treatment with 10 nM E2 compared to 1 nM E2, it certainly was not as striking as the difference in E2F1 induction after treatment with 100 pM versus 10 nM R5020. Thus, PR appears to be unique in its ability to regulate E2F1 expression in a dose-dependent biphasic manner.
Figure 3.3: Comparison of dose response curves for transcriptional activation of E2F1 by PR, ER or AR

Synchronized T47D:A18 cells were treated with increasing concentrations of a PR ligand (R5020), an ER ligand (estradiol, E2) or an AR ligand (dihydrotestosterone, DHT) for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P (positive control PR target gene), PS2 (positive control ER target gene), FKBP51 (positive control AR target gene) or 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. Preliminary data (n=1).

3.2.1.1.5 Conditions under which low-dose progestins elicit maximal E2F1 transcription

3.2.1.1.5.1 Growth factor availability

Our next goal was to define the conditions under which PR is able to regulate E2F1 transcription in a biphasic manner. First, we examined the environmental context in
which we have observed PR-mediated induction of maximal E2F1 transcription after treatment with low-dose progestins. For our qPCR studies, T47D:A18 cells are seeded in charcoal-stripped, phenol red-free Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 8% charcoal-stripped fetal bovine serum (CS-FBS). The process of charcoal-stripping is used to remove steroid hormones from media. Cells are grown in this steroid-free, nutrient-rich environment for 48 h, then switched to media containing 0.1% CS-FBS, which synchronizes the cells by inducing G₀ arrest. After 24 h of serum-starvation, cells are treated with hormone for the appropriate time period and harvested for qPCR analysis.

To explore whether growth factor availability affects progestin induction of E2F1 mRNA levels, we modified the media conditions that T47D:A18 cells are grown in prior to treatment with progestins. To do this, we removed the serum-starvation step, and instead cultured T47D:A18 cells in charcoal-stripped DMEM plus 8% CS-FBS for 72 h prior to ligand treatment. Intriguingly, the dose response curve for progestin-mediated transcriptional activation of E2F1 that we observed under these conditions was quite different than in previous experiments. Without the serum-starvation step, maximal induction of E2F1 mRNA levels occurs after treatment with high-dose progestins, and there is no longer a spike in E2F1 transcriptional activation after treatment with low-dose 100 pM R5020 (personal communication from Dr. S. Kobayashi, Duke University, Durham, NC).
3.2.1.1.5.2 Alternative PR-positive cell lines

In most breast cancer cell lines, PR activity is intrinsically linked to the estrogen receptor (ER) because it is necessary to treat with estrogens in order to induce sufficient PR expression. However, ER has previously been shown to induce expression of E2F1, and we wanted to focus solely on PR-specific regulation of E2F1 expression. We chose T47D cells as a model system for the majority of our studies because this cell line exhibits ER-independent, constitutive expression of both PR-A and PR-B (82, 195). However, we did want to explore progestin regulation of E2F1 transcription in other PR-positive cell lines, in order to determine whether the biphasic induction of E2F1 elicited by R5020 in T47D:A18 cells is an isolated phenomenon restricted to a single cell line.

Since studies in the previous chapter confirmed that the PR-B isoform is both necessary and sufficient for progestin-mediated induction of E2F1 gene expression, we first assessed progestin regulation of E2F1 transcription in T47D:C42 cells that stably express wild-type PR-B alone. T47D:C42-PR-B cells were treated with increasing amounts of R5020 for 18 h. The resulting dose response curve for transcriptional activation of E2F1 was not biphasic (Figure 3.4), but instead resembled the traditional dose response curve for the classical PR target gene S100P. Similar results were seen in BT483 cells, an ER-positive, PR-positive breast cancer cell line. Our qPCR analysis shows that treatment of BT483 cells with low-dose 100 pM R5020 was able to stimulate a significant induction of E2F1 mRNA levels, but the response was not suppressed at higher doses of R5020 (Figure 3.5).
Synchronized T47D:C42 cells expressing wild-type hPR-B alone were treated with increasing concentrations of R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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. Preliminary data (n=1).

Synchronized BT483 cells were treated with increasing concentrations of R5020 for 18 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. S100P or 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=2).
3.2.1.1.6 Mechanisms by which low-dose progestins induce maximal levels of E2F1 mRNA

Thus far, we have established that when T47D:A18 breast cancer cells are grown under serum-starved conditions, treatment with low-dose R5020 or progesterone induces higher mRNA levels of certain PR target genes than treatment with high-dose progestins. However, the molecular mechanisms behind this biphasic regulation remain unclear, so we performed studies to explore the specific effects of low-dose versus high-dose progestins on downstream pathways by which PR controls target gene expression. We utilized PR induction of E2F1 expression as our model system, since the regulatory mechanisms by which PR modulates E2F1 expression were extensively characterized in the previous chapter.

3.2.1.1.6.1 Direct regulation of E2F1 transcription

We began by assessing the direct recruitment of PR to enhancer elements surrounding E2F1 after treatment with low-dose versus high-dose R5020. Recruitment of PR to a previously characterized intronic PRE within FKBP51 was used as a positive control for PR binding (100). In the previous chapter, we showed that ligand-bound PR associates very strongly with Distal Site #4; our current ChIP analyses showed that treatment with low-dose 100 pM R5020 for 1, 2 or 18 h stimulates a small increase in PR recruitment to Distal Site #4, albeit much weaker than that elicited by high-dose 10 nM R5020 (Figure 3.6). A similar pattern of PR recruitment was observed for two other “strong” enhancer sites (Distal Sites #5-6, data not shown). In contrast, for enhancer sites
such as Proximal Site #1 (Figure 3.6) or Distal Site #3 (data not shown), which were previously shown to be weak sites of PR recruitment after treatment with 10 nM R5020, we did not observe any recruitment of PR after treatment with low-dose 100 pM R5020.

![Figure 3.6: Recruitment of PR to enhancer elements nearby E2F1](image)

Synchronized T47D:A18 cells were treated with vehicle (veh), low-dose 100 pM R5020 (R), or high-dose 10 nM R5020 (R) for the indicated time points. Cells were harvested after cross-linking and subjected to immunoprecipitation with either mouse IgG control (mlgG) or PR antibody. Following reversal of cross-linking, DNA was isolated and subjected to qPCR analysis using primers spanning a region in FKBP51 (positive control), stromelysin (negative control), or PR-binding enhancer regions surrounding E2F1 (Proximal Site #1, Distal Site #4). The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment (n=2).
3.2.1.6.2 Hyperphosphorylation of Rb and recruitment of E2F1 to the E2F1 promoter

Given that treatment with low-dose progestins did not seem to enhance direct PR recruitment to the enhancer elements surrounding E2F1, we next hypothesized that treatment with low-dose versus high-dose progestins might have differential effects on indirect pathways by which PR regulates transcriptional activation of target genes. In the previous chapter, we established that one of the mechanisms by which PR induces the transcriptional activation of E2F1 is by increasing the hyperphosphorylation of Rb at later time points, which results in increased recruitment of E2F1 to its own promoter and thereby activates a positive feedback loop that further amplifies transcription of E2F1. To determine whether varying doses of R5020 have differential impacts on the hyperphosphorylation of Rb, we assessed the phosphorylation status of Rb in T47D:A18 cells treated with low-dose or high-dose R5020 for 18 h. Preliminary Western blot analysis with total and phospho-specific Rb antibodies shows that treatment with low-dose 100 pM R5020 led to a significant increase in total levels of hyperphosphorylated Rb and specific phosphorylation of Rb at Ser^{807/811}, as well as a small increase in phosphorylation of Rb at Ser^{780} (Figure 3.7). In stark contrast, the levels of hyperphosphorylated Rb and Rb phosphorylated at Ser^{780} or Ser^{807/811} in cells treated with high-dose 10 nM R5020 remain identical to those in vehicle-treated cells (Figure 3.7).
Synchronized T47D:A18 cells were treated with vehicle (veh), low-dose 100 pM R5020 or high-dose 10 nM R5020 for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total Rb, Rb phosphorylated on Ser780 (p-Rb Ser 780), or Rb phosphorylated on Ser807/811 (p-Rb Ser 807/811). pp-Rb = hyperphosphorylated Rb, ns = non-specific band. Preliminary data (n=1).

Since treatment with high-dose R5020 was unable to stimulate hyperphosphorylation of Rb, we hypothesized that the subsequent progestin-mediated increase in recruitment of E2F1 to its own promoter might correspondingly be reduced. To address this question, we performed ChIP experiments in T47D:A18 cells to measure E2F1 occupancy at its own promoter after treatment with low-dose versus high-dose R5020. Treatment with low-dose or high-dose R5020 for 2 h did not result in a significant increase in E2F1 recruitment to the region of the E2F1 promoter containing E2F binding sites (Figure 3.8). By 10 h post-treatment, a small increase in E2F1 recruitment to its own promoter was evident, but there was no difference between low-dose versus high-dose progestin treatment. There was a significant increase in the level of
E2F1 recruitment to the E2F1 promoter between 10 and 18 h of treatment with low-dose 100 pM R5020; however, the level of E2F1 recruitment to the E2F1 promoter after treatment with high-dose 10 nM R5020 did not change between 10 and 18 h (Figure 3.8).

**Figure 3.8: Recruitment of E2F1 to E2F binding sites within its own promoter**

Synchronized T47D:A18 cells were treated with vehicle (veh), low-dose 100 pM R5020, or high-dose 10 nM R5020 for the indicated time points. Cells were harvested after cross-linking and subjected to immunoprecipitation with either mouse IgG control (mlgG) or E2F1 antibody. Following reversal of cross-linking, DNA was isolated and subjected to qPCR analysis using primers spanning a region in the E2F1 promoter containing E2F binding sites. The results are presented as percent input ± SEM for triplicate amplification reactions from one representative experiment (n=2).
Thus, we have determined that low-dose and high-dose progestins do not have identical effects on the regulatory pathways by which PR controls target gene expression. With regards to direct regulation, treatment with low-dose progestins stimulated less recruitment of PR to enhancer elements surrounding E2F1 than treatment with high-dose progestins. Conversely, low-dose progestins were better able to activate indirect pathways of E2F1 regulation. Treatment with low-dose R5020 elicited hyperphosphorylation of Rb, whereas high-dose R5020 did not, and subsequent recruitment of E2F1 to its own promoter was somewhat increased in T47D:A18 cells treated with low-dose versus high-dose R5020.

3.2.1.2 Transrepression of ER-mediated transcriptional activation

The increased stimulation of PR target gene transcription after treatment with low-dose progestins prompted us to question whether using lower amounts of R5020 might also enhance other downstream activities of PR, such as transrepression of estrogen-mediated transcriptional activation of ER target genes (196). However, qPCR analysis demonstrated that treatment with low-dose progestins did not improve the ability of PR to transrepress ER actions. In fact, treatment of T47D:A18 cells with low-dose 100 pM R5020 did not repress ER-mediated induction of pS2 mRNA levels as efficiently as treatment with high-dose 10 nM R5020 (Figure 3.9).
Synchronized T47D:A18 were treated for 18 h with vehicle (veh), 1 nM estradiol (E2) or the indicated concentrations of R5020 (R), or a combination of both as indicated. After treatment, cells were lysed and RNA was isolated and reverse transcribed. PS2 (ER target gene) mRNA levels were quantified using qPCR and normalized to the housekeeping gene 36B4. Results are expressed as fold induction over vehicle-treated cells ± SEM. Preliminary data (n=1).

3.2.1.3 Regulation of S-phase entry

Progestins have previously been shown to have proliferative effects in breast cancer cells (26), so we were intrigued by the observation that the subset of genes that are maximally induced by low-dose progestins in T47D:A18 breast cancer cells includes key regulators of cell cycle such as cyclin D1 and E2F1. Before embarking on the analysis of low-dose R5020 effects on cell cycle, we verified that the previously observed biphasic induction of E2F1 mRNA levels by progestins also translates to a similar pattern of regulation at the protein level. Western blot analysis confirmed a biphasic dose response.
curve of E2F1 expression, wherein treatment of T47D:A18 cells with low-dose 100 pM R5020 for 18 h induced significantly higher levels of E2F1 protein than high-dose 10 nM R5020 (Figure 3.10).

![Figure 3.10: Maximal induction of endogenous E2F1 protein by low-dose R5020](image)

Synchronized T47D:A18 cells were treated with vehicle (veh) or increasing concentrations of R5020 for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for E2F1 or GAPDH as a loading control. A representative blot is shown (n=2).

To investigate whether treatment with low-dose progestins can enhance PR-mediated proliferation, we utilized a BrdU (bromodeoxyuridine) incorporation assay to measure the number of cells entering S-phase after treatment with increasing concentrations of R5020. Prior to treatment, T47D:A18 cells were cultured in one of the following conditions, ranging from nutrient-poor to nutrient-rich: (1) a serum-starved environment, (2) a steroid-free, full serum environment, or (3) a full serum environment.

To our surprise, the preliminary data showed that despite a dramatic induction of E2F1 protein under serum-starved conditions (Figure 3.10), treatment with R5020 was unable to stimulate any significant change in S-phase entry (Figure 3.11).
Figure 3.11: Progestin-mediated induction of S-phase entry

T47D:A18 cells were grown in a serum-starved environment (0.1% charcoal-stripped fetal bovine serum, CS-FBS), a steroid-free full serum environment (10% CS-FBS), or a full serum environment (10% FBS). Next, cells were treated for 18 h with vehicle (veh), increasing concentrations of R5020, or FBS. Cells were harvested and assayed for BrdU incorporation per manufacturer’s protocol. Results are expressed as fold induction over vehicle-treated cells (grown in the serum-starved environment). Preliminary data (n=1).

Conversely, in cells grown in either of the nutrient-rich conditions, treatment with low-dose 100 pM R5020 was sufficient to induce a significant increase in S-phase entry compared to vehicle-treated cells. However, the dose response curves for S-phase entry did not resemble the biphasic pattern of progestin regulation that was observed for E2F1 expression. Maximal induction of S-phase entry occurred after treatment with 1 nM R5020, and there was no significant difference in S-phase entry between cells treated
with low-dose 100 pM R5020 or high-dose 10 nM R5020 (Figure 3.11). Thus, the increased induction of E2F1 and other regulators of cell cycle by low-dose R5020 does not appear to enhance progestin-mediated proliferation of breast cancer cells.

**3.2.2 The differential effects of low-dose versus high-dose progestins on phosphorylation of PR and receptor turnover**

To ascertain whether treatment with varying doses of progestins had differential effects on the expression of PR itself, we examined the expression of total PR in T47D:A18 cells treated with increasing concentrations of R5020 for 18 h. Intriguingly, while total levels of PR did not appear to change, we observed an upward shift in the mobility of PR after treatment with 1 or 10 nM R5020, which was not apparent after treatment with lower doses of R5020 (Figure 3.12). Interestingly, this upshift of PR corresponded to a decreased ability of R5020 to induce E2F1 expression.

![Progestin-mediated changes in the electrophoretic mobility of PR](image)

**Figure 3.12: Progestin-mediated changes in the electrophoretic mobility of PR**

Synchronized T47D:A18 cells were treated with vehicle (veh) or increasing concentrations of R5020 for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total PR, E2F1 or GAPDH as a loading control. A representative blot is shown (n=2).
3.2.2.1 Phosphorylation of PR

It has previously been established that the characteristic upward shift in electrophoretic mobility of PR on sodium dodecyl sulfate (SDS) gels after treatment with progestins is due to post-translational modifications such as phosphorylation, which result in an increase in the apparent molecular weight of both PR-A and PR-B (170). Since treatment with low-dose 100 pM R5020 was unable to stimulate the upshift in PR mobility (Figure 3.12), we hypothesized that low-dose progestins could be failing to trigger phosphorylation of PR. Therefore, we decided to investigate the phosphorylation status of PR at multiple sites after treatment with low-dose or high-dose progestins.

The N-terminal domain of PR contains at least seven serine (Ser) residues that can serve as phosphorylation sites (216-218). We were able to obtain three phosphoserine site-specific monoclonal antibodies, which recognize PR that is phosphorylated at Ser\textsuperscript{162}, Ser\textsuperscript{190}, or Ser\textsuperscript{294} (27, 129). Both Ser\textsuperscript{162} and Ser\textsuperscript{190} are basally phosphorylated, hormone-inducible sites, whereas phosphorylation of PR at Ser\textsuperscript{294} is hormone-dependent. Ser\textsuperscript{162} is unique to PR-B, as it is located within the N-terminal segment that is missing in PR-A; conversely, Ser\textsuperscript{190} and Ser\textsuperscript{294} are located within an N-terminal region shared by PR-A and PR-B. Although Ser\textsuperscript{294} is present in both PR isoforms, it appears to be preferentially phosphorylated on PR-B; Edwards and coworkers speculate that the distinct conformation of the N-terminal domains of PR-A may inhibit phosphorylation of this site (27). Finally, in vitro studies have shown that Cyclin A-cyclin-dependent kinase 2
(CDK2) phosphorylates PR at Ser$^{162}$ and Ser$^{190}$, whereas Ser$^{294}$ is a target of the MAPK pathway (89, 129, 169, 216).

To compare the effects of low-dose versus high-dose progestins on PR phosphorylation, we utilized the aforementioned phospho-specific antibodies to examine the phosphorylation of PR at Ser$^{190}$, Ser$^{294}$, and Ser$^{162}$ in T47D:A18 cells treated with increasing amounts of R5020 for 18 h. Western blot analysis revealed a slight increase in phosphorylation of PR at Ser$^{190}$ and Ser$^{162}$ after treatment with high-dose R5020 compared to low-dose R5020, but the most striking difference in PR phosphorylation after treatment with varying amounts of R5020 was observed for phosphorylation of PR at Ser$^{294}$ (Figure 3.13). While treatment with high concentrations of R5020 such as 1 or 10 nM induced significant phosphorylation of PR-B at Ser$^{294}$, no phosphorylation at this site was apparent after treatment with low-dose 100 pM R5020.
Figure 3.13: Induction of specific PR phosphorylation events by progestins

Synchronized T47D:A18 cells were treated with vehicle (veh) or increasing concentrations of R5020 for 18 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total PR, PR phosphorylated at Ser190 (p-PR Ser190), PR phosphorylated at Ser294 (p-PR Ser294) or PR phosphorylated at Ser162 (p-PR Ser162). A representative blot is shown (n=2).

Since the change in phosphorylation status of PR at Ser\textsuperscript{294} after treatment with 100 pM versus 1 nM R5020 was so abrupt, we carried out a more detailed dose response experiment to ascertain the exact concentration of R5020 that is required to stimulate this phosphorylation event. T47D:A18 cells were treated with a range of R5020 concentrations between 100 pM to 1 nM for 18 h. Western blot analysis confirmed that
phosphorylation of PR at Ser\textsuperscript{294} is completely absent after treatment with 100 pM R5020, but levels of PR phosphorylated at Ser\textsuperscript{294} gradually increase as the dosage of R5020 increases from 250 pM to 1 nM (Figure 3.14A). Similar results were seen in T47D:A18 cells treated with a range of R5020 concentrations between 100 pM to 1 nM for the shorter time period of 7 h (Figure 3.14B). Interestingly, we noted that treatment with low-dose 100 pM R5020 for 7 h was sufficient to induce a significant increase in phosphorylation of PR at Ser\textsuperscript{162} (Figure 3.14B), a phenomenon that was not observed after a longer 18 h treatment (Figure 3.13).
Figure 3.14: Detailed dose response curves for induction of PR phosphorylation by progestins

Synchronized T47D:A18 cells were treated with vehicle (veh) or increasing concentrations of R5020 for (A) 18 h or (B) 7 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total PR, PR phosphorylated at Ser190 (p-PR Ser190), PR phosphorylated at Ser294 (p-PR Ser294), PR phosphorylated at Ser162 (p-PR Ser162) or α-tubulin or β-tubulin as a loading control. Preliminary data (n=1).

3.2.2.2 Degradation of PR

Previous studies have demonstrated that the phosphorylation of PR on Ser$_{294}$ plays an essential role in receptor turnover. Treatment with R5020 stimulates PR phosphorylation by MAPKs on Ser$_{294}$, which subsequently leads to rapid degradation of PR via the ubiquitin-proteasome pathway (89). Since our studies showed that treatment
with low-dose R5020 failed to stimulate phosphorylation of PR at Ser\textsuperscript{294}, we hypothesized that low-dose progestins might correspondingly fail to elicit receptor turnover. To address this question, we examined the expression levels of PR in T47D:A18 cells treated with a range of R5020 concentrations for various extended time periods from 18 to 48 h. Western blot analysis shows that high-dose 1 nM R5020 stimulates robust levels of PR phosphorylated at Ser\textsuperscript{294} by 18 h post-treatment, and this corresponds to a subsequent reduction in total PR levels at 24 h, with almost complete receptor degradation occurring by 48 h (Figure 3.15).

![Figure 3.15: Progestin-mediated induction of E2F1 expression, phosphorylation of PR and receptor turnover](image)

Synchronized T47D:A18 cells were treated with vehicle (veh) or increasing concentrations of R5020 for the indicated time periods. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total PR, PR phosphorylated at Ser294 (p-PR Ser294) or E2F1. A representative blot is shown (n=2).

In contrast, treatment with low-dose 100 pM R5020 fails to induce phosphorylation of PR at Ser\textsuperscript{294} at any time point post-treatment. Consequently,
degradation of PR after treatment with low-dose progestins occurs at a much slower rate, with substantial levels of total PR remaining at 48 h post-treatment. While low-dose 100 pM R5020 did not induce phosphorylation of PR at Ser294 or efficient receptor degradation, it did induce maximal expression of E2F1 as late as 36-48 h post-treatment, the latest time points that we assessed (Figure 3.15). However, treatment with high-dose progestins failed to elicit this “long-term” induction of E2F1 expression; in fact, E2F1 protein levels were almost back down to basal levels by 48 h post-treatment with high-dose 1 nM R5020.

3.3 Discussion

In this investigation, we sought to identify and characterize any differential effects of low-dose versus high-dose progestins on the downstream activities of PR. Specifically, we found differences between the consequences of low-dose versus high-dose progestin treatments for the transcriptional activation of a subset of PR target genes, for the induction of E2F1 protein levels, for PR-mediated hyperphosphorylation of Rb, and for the phosphorylation and subsequent degradation of PR itself. These discoveries may be clinically significant, given the widespread use of PRMs in contraceptives, HRT, and endocrine therapy for breast cancer.

3.3.1 Biological consequences of treatment with low-dose progestins

For classic PR target genes such as S100P, treatment with increasing amounts of R5020 stimulates a corresponding increase in mRNA levels, until a plateau of maximal
transcription is reached at high doses of 1 to 10 nM R5020 (Figure 3.1). However, we observed a biphasic dose response curve for progestin-mediated transcriptional activation of other PR target genes. In particular, we demonstrated that treatment of T47D:A18 cells with low-dose R5020 or progesterone led to maximal expression of a subset of PR target genes, including cyclin D1 and E2F1 (Figure 3.1 & 3.2, Table 3.1).

While enhanced induction of target gene transcription by low-dose progestins has not been previously reported, there exists a precedent for biphasic regulation of target gene expression by steroid hormones. Shao and coworkers demonstrated that treatment of LNCaP prostate cancer cells with the synthetic androgen methyltrienolone (R1881) results in a dose-dependent biphasic effect on downstream gene expression and cell growth (167). Therefore, we were intrigued by the observation that the subset of genes that are maximally induced by low-dose progestins in T47D:A18 breast cancer cells includes key regulators of cell cycle such as cyclin D1 and E2F1 (Table 3.1); furthermore, low-dose progestins are able to efficiently stimulate hyperphosphorylation of Rb, which leads to progression of cell cycle (Figure 3.7).

Preliminary studies were conducted to investigate whether treatment with low-dose progestins can enhance PR-mediated proliferation; however, treatment of T47D:A18 cells with low-dose or high-dose R5020 was equally sufficient to stimulate an increase in S-phase entry (Figure 3.11). Lange and coworkers have proposed that treatment with progestins may serve to “prime” breast cancer cells for cross talk with proliferative signals from other growth factors (88). Although we did not observe any difference in
progestin-mediated proliferation in cells treated with low-dose versus high-dose progestins, we have yet to assess the proliferative response of these cells to a secondary treatment with epidermal growth factor (EGF) or serum. Further studies are definitely warranted to determine whether the actions of low-dose progestins impact downstream PR biology by increasing the proliferative potential of breast cancer cells.

Interestingly, we noted that a biphasic dose response curve for transcriptional activation of E2F1 was only observed in T47D:A18 cells grown in specific media conditions. Studies are ongoing to ascertain why biphasic regulation of E2F1 expression by progestins was seen in cells exposed to 24 h of serum-starvation (Figure 3.1), and not in cells grown in a more nutrient-rich environment (personal communication from Dr. S. Kobayashi). One possible hypothesis is that serum contains some factor that counteracts the ability of low-dose progestins to enhance induction of E2F1 expression. In addition, while we did confirm that PR activates E2F1 transcription in T47D:C42 cells expressing PR-B alone and in BT483 cells, we did not observe any biphasic regulation by progestins in these cell lines (Figure 3.4 & 3.5). Further studies are necessary to determine whether biphasic regulation of target gene expression by progestins occurs in other PR-positive breast cancer cell lines and environmental milieus.

The molecular mechanisms underlying the biphasic progestin-mediated regulation of a subset of PR target genes remain unclear. Since qPCR analysis measures the steady-state level of E2F1 transcripts, we do not know whether treatment with high-dose R5020 actually stimulates less transcriptional activation compared to low-dose R5020;
alternatively, the rate of transcription may be identical, but treatment with high-dose progestins activates a secondary feedback mechanism that inhibits mRNA levels by affecting transcript stability or degradation. Another possibility is that the relative level of receptor saturation dictates the ability of PR to modulate alternative pathways that indirectly amplify transcription of certain target genes. Studies are ongoing to further investigate the nature of this biphasic regulation.

Having previously established that PR regulates E2F1 expression in both direct and indirect manners, we assessed the effects of low-dose versus high-dose progestins on these pathways. Direct regulation of E2F1 transcription by PR was not enhanced by treatment with low-dose progestins; in fact, ChIP studies revealed that treatment with 100 pM R5020 stimulated less recruitment of PR to enhancer elements surrounding E2F1 than treatment with 10 nM R5020 (Figure 3.6). However, treatment with low-dose R5020 was not sufficient to saturate the receptor with ligand; correspondingly, the total amount of agonist-bound PR in cells treated with low-dose progestins is much smaller than in cells treated with high-dose progestins. Therefore, it is possible that ChIP analysis is not sensitive enough to detect any relative increases in the recruitment of agonist-bound PR to enhancer elements after treatment with low-dose progestins compared to high-dose progestins, given the proportional difference in receptor occupancy.

In terms of indirect PR regulation of E2F1 expression, we discovered that low-dose progestins were able to stimulate the hyperphosphorylation of Rb, whereas high-dose progestins lacked this capability (Figure 3.7). However, treatment with high-dose
progestins still resulted in increased E2F1 recruitment to its own promoter, albeit to a lower extent than that induced by low-dose progestins (Figure 3.8). Additional studies are needed to further elucidate the potential mechanisms by which low-dose progestins are able to stimulate maximal transcription of E2F1.

Importantly, we observed that treatment with increasing doses had differential effects on the electrophoretic mobility of PR on SDS gels, due to differences in post-translational modifications such as phosphorylation of the receptor (Figure 3.12). While slight dose-dependent differences were noted in phosphorylation of PR at Ser\textsuperscript{162} and Ser\textsuperscript{100}, the most striking disparity was perceived in phosphorylation of PR at Ser\textsuperscript{294} (Figure 3.13). Although PR can be phosphorylated at many other Ser residues, we were only able to examine the phosphorylation of PR at sites for which we had phospho-specific antibodies. As additional phospho-specific PR antibodies become available, further progress can be made in assessing the differential effects of low-dose versus high-dose progestins on phosphorylation of PR at other sites.

Furthermore, by treating cells with low-dose 100 pM R5020, we were able to uncouple PR-mediated induction of target gene transcription from ligand-dependent receptor turnover. We showed that treatment with high-dose progestins results in phosphorylation of PR at Ser\textsuperscript{294} and efficient degradation of PR by 48 h post-treatment (Figure 3.15). In contrast, treatment with low-dose progestins does not stimulate phosphorylation of PR at Ser\textsuperscript{294} and therefore fails to induce phospho-Ser\textsuperscript{294}-dependent degradation of PR via the 26S proteasome pathway. While PR expression levels do
decline somewhat between 18 h and 48 h post-treatment with low-dose 100 pM R5020, the receptor turnover is much less efficient than that triggered by high-dose progestins (Figure 3.15). Consequently, PR activated by low-dose progestins remains present and active for longer time periods, and continues to induce significant expression of E2F1.

Intriguingly, phosphorylation of PR-B at Ser\textsuperscript{294} has been linked to ligand-dependent export of PR-B from the nucleus, as well as subsequent receptor turnover; in contrast, PR-A does not appear to be phosphorylated at this site and remains largely nuclear, though it is a much weaker transactivator than PR-B and functions primarily as a transrepressor of PR-B and other nuclear receptors (90, 146). We do not believe that increased degradation of PR is responsible for the lower induction of E2F1 expression by high-dose progestins, since the PR protein levels at 18 h post-treatment with low-dose or high-dose R5020 remain essentially the same. However, we have not evaluated the localization of each PR isoform within the cell as a function of hormone exposure. It is feasible that treatment with high-dose progestins, and subsequent phosphorylation of PR-B at Ser\textsuperscript{294}, results in the export of PR-B from the nucleus; once outside the nucleus, ligand-bound PR-B would be unable to exert any direct effects on E2F1 transcription. Furthermore, ligand-bound PR-A may then have increased access to enhancer elements around \textit{E2F1}, where it could antagonize the indirect actions of PR-B and act to repress transcription. Conversely, since treatment with low-dose progestins does not result in phosphorylation of PR-B at Ser\textsuperscript{294}, ligand-bound PR-B could remain in the nucleus and compete with PR-A for access to E2F1 enhancer elements. Thus the subcellular
localization of each PR isoform after treatment with low-dose versus high-dose progestins could be responsible for the biphasic gene regulation that we have observed. While our ChIP studies indicate that PR is present at the enhancer elements around E2F1 even at later time points such as 10 or 18 h, we have yet to attempt ChIP experiments with isoform-specific PR antibodies. It would therefore be worthwhile to determine the relative occupancy of PR-B compared to PR-A on the E2F1 enhancer elements or other target gene promoters at various time points after treatment with low-dose or high-dose progestins.

Finally, while previous research on phosphorylation of PR at Ser^{294} has focused on the link to nuclear trafficking and receptor turnover, this phosphorylation event (and others) may have additional effects on the downstream biology of PR. It is possible that phosphorylation might affect the transcriptional activity of PR by changing its ability to recruit cofactors or modulate other signaling pathways. We have shown that PR remains unphosphorylated at Ser^{294} after treatment with low-dose progestins; perhaps this opens up a receptor surface for interaction with other regulatory molecules that further amplify downstream gene expression. Conversely, phosphorylation of PR at Ser^{294} after treatment with high-dose progestins may facilitate interaction with a co-repressor that dampens the induction of target gene transcription. Thus, exploring the ramifications of these receptor phosphorylation events is an area of continued research in our group.

### 3.3.2 Clinical applications of low-dose progestins: helpful or harmful?

The differential effects that we observed after treatment with low-dose or high-
dose progestins may have important clinical ramifications, given the diverse range of medical therapies that utilize low-dose progestins. The current paradigm in both contraceptives and combination HRT is to prescribe the lowest possible dose of progestin necessary to achieve the desired clinical outcome, while hopefully minimizing the chance of serious side effects such as increased risk of breast cancer. However, our data suggest that in certain contexts, less progestin is actually having more effects on certain downstream PR activities, including the regulation of target genes involved in cell cycle control. Thus, further studies are warranted to determine whether administering low-dose progestins for contraceptive purposes or HRT is a helpful or harmful regimen.

3.3.3 Conclusion

In summary, we have identified several areas of PR biology that respond differently to treatment with low-dose versus high-dose progestins. Specifically, we have shown that treatment of breast cancer cells with low-dose progestins can induce maximal transcriptional activation of a subset of PR target genes, including the cell cycle regulators cyclin D1 and E2F1. Furthermore, low-dose and high-dose progestins have differential effects on the phosphorylation of PR and subsequent receptor turnover. Cumulatively, these findings underscore the importance of establishing the effects of a wide range of progestin concentrations on target gene expression and other PR actions, so that we are able to accurately predict the potential consequences of PRMs on downstream PR signaling pathways and biology.
4 Conclusions and Future Implications

4.1 Multimodal regulation of PR target genes

In the early years of nuclear receptor research, many studies focused on the direct effects of PR on transcriptional regulation of target genes through binding to canonical PREs and recruitment of coactivators. As data accumulated which suggested that progestins could rapidly activate other cytoplasmic signaling cascades independently of the classical nuclear actions of PR, much effort was channeled into identifying potential PR target genes that are indirectly regulated by secondary signaling pathways. However, the nuclear and extranuclear actions of PR do not occur in isolation, and a growing body of evidence has highlighted that the cross talk that occurs between PR and cytoplasmic signaling cascades is bidirectional and complex. Therefore, it is crucial that we endeavor to unravel the mechanisms by which these pathways can interact and integrate to ultimately impact gene expression.

Here, we show that PR is a component of several distinct pathways that function both directly and indirectly to positively up-regulate E2F1 expression in breast cancer cells. Firstly, PR directly regulates E2F1 transcription by binding to proximal and distal enhancer sites located near E2F1. Secondly, progestins induce the hyperphosphorylation of Rb, which results in increased recruitment of E2F1 to its own promoter, thereby activating a positive feedback loop that further amplifies its transcription. Finally, PR induces expression of KLF15 and potentially other Sp/KLF family members, which bind
to GC-rich regulatory regions within the E2F1 promoter and further activate transcription.

Our model of multimodal regulation of E2F1 expression by progestins highlights the importance of considering how multiple nuclear receptor signaling pathways may converge to regulate transcription of a single target gene. Studying the effects of a single PR signaling pathway on E2F1 expression would have resulted in an incomplete view of PR action, since the combined actions of each component pathway are required for maximal progestin-mediated up-regulation of E2F1 transcription. We believe that this model of PR cooperation with secondary regulatory factors represents a much more up-to-date, realistic paradigm of nuclear receptor action than the original model of direct PR actions on target gene promoters. In fact, given the large overlap in occurrence of putative binding sites for PR, Sp family members and E2F1 in the promoter regions of PR target genes identified in our microarray analysis, we intend to further explore the co-regulation of PR target genes at a global level. For instance, large-scale knockout studies in which transcription factors such as E2F1 or KLF15 are depleted should allow us to construct a hierarchical map that illustrates the complex interactions between PR and these secondary factors in the overall regulation of target gene expression.

In this particular example of PR target gene regulation, E2F1 and KLF15 act to amplify the initial stimulatory effects of PR on E2F1 transcription; however, in other contexts, these secondary cofactors and others may function in a negative feedback loop to repress further expression of a gene product that is no longer needed. Previous
microarray studies have demonstrated that treatment with progestins leads to different gene expression profiles in human breast cancer cells compared to endometrial carcinoma cells (150, 172). The multimodal regulation shown herein may provide one explanation for this tissue-specific PR regulation; that is, progestins may be able to activate distinct transcriptional programs due to differential availability of a diverse network of secondary transcription factors, such as members of the E2F or Sp/KLF families, which can subsequently function to amplify or antagonize PR actions in a context-specific manner.

Finally, the exquisite, multi-level regulation that we observed would suggest that progestin up-regulation of E2F1 protein levels is involved in a downstream PR biology that serves an important physiological function. The findings presented here focused on the molecular mechanisms underlying PR regulation of E2F1 expression; however, given the diverse regulatory roles of E2F1 in key cellular processes such as proliferation and apoptosis, future studies should be undertaken to investigate the impact of E2F1 up-regulation event on these biological processes.

4.2 **Differential effects of low-dose versus high-dose progestins**

Throughout the female reproductive cycle, circulating levels of progesterone fluctuate dramatically from less than 2 ng/mL up to 20 ng/mL (209). During the follicular phase, while estrogen levels are predominant, PR does not act to antagonize the proliferative effects of ER in the uterus. However, when progesterone levels rise throughout the luteal phase, PR suppresses proliferation and promotes decidualization.
Given this physiological pattern, it would make sense if PR were designed to react differently when stimulated with differing amounts of progesterone.

Furthermore, data from various clinical trials may support a dose-dependent role for progestins and PR in biological processes involved in breast carcinogenesis. On one hand, data from the WHI and HABITS trials of combination HRT have suggested that low-dose progestins may increase a woman’s risk for breast cancer (105, 154). Conversely, endocrine therapy with high-dose progestins can serve as an effective front-line and second-line treatment for advanced, metastatic breast cancer (128). While the putative role of exogenous progestins as a breast cancer risk factor remains somewhat controversial, many investigators have focused on characterizing the effects of progestins on the in vitro proliferation of breast cancer cells such as T47D cells. However, the vast majority of these experiments have been performed using high concentrations of progestins, and our data suggest that the conclusions drawn from these studies may not hold true for all concentrations of progestins, as previously assumed.

Here, we found that treatment with lower doses of R5020 induces maximal expression of key cell cycle regulators, including E2F1 and cyclin D1; it would not be surprising if this differential regulation leads to different biological consequences than those observed after treatment with high-dose progestins. It is therefore crucial that we broaden our experimental scope beyond the activities of PR at the molecular level, and endeavor to assess the effects of low-dose progestins on physiological processes such as proliferation. Although preliminary BrdU incorporation assays did not reveal any
differences in S-phase entry in cells treated with low-dose or high-dose R5020, we intend to carry out further studies to thoroughly explore whether low-dose progestins can differentially affect the immediate proliferation or relative proliferative potential of breast cancer cells. Studies should also be undertaken to investigate the effects of low-dose versus high-dose progestins on other biological endpoints relevant to breast cancer such as migration and invasion.

In order to identify specific PR biologies that are differentially regulated by low-dose or high-dose progestins, we could conduct a large-scale microarray analysis to examine genome-wide changes in PR-dependent gene transcription after treatment of T47D breast cancer cells with increasing doses of R5020. Given the differences that we have already uncovered in gene regulation in a small panel of PR target genes, it would not be surprising to see very different global expression profiles in cells treated with low-dose versus high-dose R5020. Subsequent GO-term analysis would allow us to pinpoint individual biological processes that are specifically targeted by low-dose or high-dose progestins, and subsequently follow up on any differentially-regulated biological endpoints that are especially relevant to breast cancer, such as migration or invasion.

Importantly, we were able to uncouple PR-mediated induction of target gene transcription from ligand-dependent receptor turnover. When a cell is exposed to high levels of progesterone, it acts as the “gas” that powers PR actions on downstream biology; however, ligand-dependent phosphorylation of PR at Ser\textsuperscript{294} and subsequent receptor turnover are the “brakes” that function as a negative feedback loop to safely
bring PR signaling to a halt. We showed that treatment of T47D breast cancer cells with low-dose progestins induced maximal expression of E2F1 for long time periods, but did not stimulate phosphorylation of PR at Ser\textsuperscript{294} and therefore failed to induce phospho-Ser\textsuperscript{294}-dependent degradation of PR via the 26S proteasome pathway. Thus, our data indicate that low concentrations of progestins are able to hit the gas – but unfortunately, the brake lines have been cut, which could result in a serious “accident”!

In conclusion, our data suggest that in certain contexts, less progestin is actually having more effects on certain downstream PR activities. If our “less is more” hypothesis continues to hold and extends to PR biology as well as molecular mechanisms of target gene regulation, it would have critical implications for the field of endocrine therapy, due to the diverse range of therapeutics that contain low-dose progestins. Given the WHI and HABITS data, the current paradigm in both contraceptives and combination HRT is to prescribe the lowest possible dose of progestin necessary to achieve the desired clinical outcome, while hopefully minimizing the chance of serious side effects such as increased risk of breast cancer. However, our studies indicate that administering low-dose progestins for contraceptive purposes or HRT may actually be a harmful regimen for women in general, and especially those who are already at a higher risk for breast cancer.
5 Materials and Methods

5.1 Biochemicals

Promegestone (R5020) was purchased from NEN Life Science Products (Boston, MA) and dihydrotestosterone (DHT) was obtained from Steraloids (Newport, RI). 17β-estradiol (E2), dexamethasone (Dex), progesterone (P4), cycloheximide (CHX), U0126 and Mithramycin A (MitA) were obtained from Sigma (St. Louis, MO).

PCR and qPCR reagents were obtained from Bio-Rad (Hercules, CA), Qiagen (Valencia, CA), Integrated DNA Technologies (Coralville, IA), and Sigma.

5.2 Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse monoclonal antibodies against E2F1 (KH95), β-tubulin (D-10), and Gal4DBD (sc-510); goat polyclonal antibodies against GAPDH (V-18) and α-tubulin (E-19); and the rabbit polyclonal GR (P-20) antibody. The mouse monoclonal antibody against total PR (1294) and the phospho-serine-specific mouse monoclonal antibodies against p-PR Ser162, p-PR Ser190, and p-PR Ser294 were kindly provided by D. Edwards (Baylor College of Medicine, Houston, TX). The mouse monoclonal HisG antibody (used to probe for His-tagged KLF15) was from Invitrogen. The mouse monoclonal total Rb (4H1) antibody and rabbit polyclonal antibodies against p44/42 MAPK (Erk1/2), phospho-Rb Ser780, and phospho-Rb Ser807/811 were all from Cell Signaling Technology (Danvers, MA). The anti-mouse-HRP, anti-rabbit-HRP and anti-
goat-HRP secondary antibody conjugates were from Amersham Biosciences (Buckinghamshire, UK).

5.3 Plasmids

5.3.1 Expression Plasmids

pBKC-hPR-B was previously described (49), and pcDNA3 was purchased from Invitrogen (Carlsbad, CA). pcDNA3-hPR-B was constructed as follows: a BamHI fragment of hPR-B (aa 22-933) was cut out from pBKC-hPR-B and subcloned into pcDNA3 using the BamHI site to create pcDNA3-PR 22-933. Next, the 5' region of PR was amplified using SV40-hPR-B (47) as a template, using the sense primer 5’-GGGGTACCCCGGCGCGCCATGACTGAGCTGAAG-3’ and the antisense primer 5’-AGGCCGGGAGCAGCAGCT-3’. This fragment was subsequently digested with KpnI and BstEII, and then cloned into pcDNA3-PR 22-933 using KpnI/BstEII sites to create pcDNA3-hPR-B. pENTR-hPR-B was constructed by cloning a KpnI to EcoRI fragment of pcDNA3-hPR-B into the pENTR-1A vector, purchased from Invitrogen. pENTR-hPR-B-C587A was created as follows: the fragment of hPR-B between the AscI and HindIII restriction sites was amplified by PCR using the PR DNA-binding mutant hPR-Bcys (a kind gift of K. Horwitz, University of Colorado, Denver, CO) as a template, using the sense primer 5’-TGCATCCTGTACAAAGCGGAGGG-3’ and the antisense primer 5’-ACTTGAAGCTTGACAAAATCTCTGTGG-3’. This fragment was cloned into pENTR-hPR-B using the AscI and HindIII restriction sites. MSCV-GWb-Gal4DBD-IRES-EGFP, MSCV-GWb-hPR-B-IRES-EGFP and MSCV-GWb-hPR-B-C587A-IRES-
EGFP were created using the Invitrogen Gateway recombinase subcloning system according to the manufacturer’s instructions. To do this, Gal4DBD, hPR-B or hPR-B-C587A were shuttled from pENTR-Gal4DBD, pENTR-hPR-B or pENTR-hPR-B-C587A to MSCV-IRES-EGFP that was converted to a Gateway destination vector.

The KLF15 expression constructs pcDNA-hKLF15 and pcDNA-hKLF15-NA291 have been previously described (136).

5.3.2 Reporter Constructs

The empty vector control pGL2-TATA-Luc and the progesterone-responsive MMTV-Luc luciferase reporter constructs have been previously described (61, 200). The normalization vector pCMVß-gal was obtained from Clontech (Palo Alto, CA). The E2F1 promoter luciferase constructs pGL2-hE2F1-Luc (-242), (-204) and (-122) have been previously described (78).

5.4 Mammalian Cell Culture

The T47D and BT483 human breast ductal carcinoma cell lines and the H4IIE rat hepatoma cell line were all purchased from American Type Culture Collection (Manassas, VA). Human mammary epithelial cells (HMECs; 63NP1) were a kind gift from J. Marks (Duke University, Durham, NC). The T47D:A18 cell line was kindly provided by V. Jordan (Fox Chase Cancer Center, Philadelphia, PA) and has been previously described (122). The PR-negative T47D:C42 cell lines stably expressing
LacZ, PR-A, PR-B, or PR-BmPro were a kind gift from D. Edwards (Baylor College of Medicine, Houston, TX), and have been previously described (15).

To create T47D:C42-Gal4DBD, T47D:C42-hPR-B and T47D:C42-hPR-B-C587A stable cell lines, parental T47D:C42 cells from D. Edwards were infected with retrovirus expressing MSCV-GWb-Gal4DBD-IRES-EGFP (negative control), MSCV-GWb-hPR-B-IRES-EGFP or MSCV-GWb-hPR-B-C587A-IRES-EGFP. EGFP positive cells were then selected through two rounds of cell sorting using flow cytometry and hPR-B expression levels were confirmed by Western blot analysis.

Unless otherwise noted, all media and supplements were purchased from Invitrogen. The T47D, T47D:A18 and BT483 cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 0.1 mM non-essential amino acids (NEAA) and 1 mM sodium pyruvate (NaPyr). T47D:C42 cell lines from D. Edwards were cultured in CellBIND tissue culture flasks (Corning, Lowell, MA) using 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, 60 µg/mL insulin, and 200 µg/mL Zeocin. T47D:C42 stable cell lines created in our lab were maintained in MEM supplemented with 8% FBS, 10 mM HEPES, 25 µg/mL gentamicin, 50 U/mL Pen/Strep, and 0.1 mM NEAA. HMECs were maintained in Mammary Epithelial Basal Medium (MEBM) (Lonza, Basel, Switzerland) supplemented with Mammary Epithelial Cell Growth Medium (MEGM) SingleQuots (Lonza), 5 µg/mL Transferrin (Sigma-Aldrich),
and 10 µM Isoproterenol (Sigma-Aldrich). H4IIE cells were maintained in MEM supplemented with 8% FBS, 0.1 mM NEAA, and 1 mM NaPyr.

All cell lines were grown in a 37°C incubator with 5% CO₂.

5.5 Transient Transfection Assays

For reporter gene assays, T47D:A18 cells were seeded in 24-well plates in phenol red-free DMEM containing 8% charcoal-stripped fetal bovine serum (CS-FBS) (Hyclone, Logan, UT), 0.1 mM NEAA and 1 mM NaPyr 24 h before transfection. DNA was introduced into the cells using Lipofectin (Invitrogen)-mediated transfection as described by the manufacturer. Briefly, triplicate transfections were performed using 3 µg of total DNA; within each experiment, the total amount (in µg) of DNA used to transfect each plate was kept constant by addition of the corresponding empty expression vector DNA lacking a cDNA insert. Cells were incubated with the DNA-Lipofectin mixture for 24 h. Next, the transfection mix was replaced with fresh media containing the appropriate ligands. Following overnight treatment, luciferase and β-galactosidase (β-gal) activities were assayed on a Fusion Alpha-FP HT Universal Microplate Reader (Perkin Elmer, Danvers Grove, IL). Results are expressed as relative luciferase activity (normalized to β-gal for transfection efficiency) for one representative experiment performed in triplicate, and error bars indicate the standard error of the mean (SEM) for the triplicate wells.
5.6 Microarray Analyses

5.6.1 T47D Cells

Oligonucleotide microarray analysis was conducted on 2 biological replicate cultures of T47D cells. For each biological replicate, T47D cells were seeded into one well of a 6-well plate per treatment in phenol red-free DMEM supplemented with 10% CS-FBS for 72 h. Cells were pre-treated for 10 min with vehicle or 10 µM U0126, then treated for 24 h with vehicle or 10 nM R5020. After treatment, the culture media was removed from each of the wells and the entire plate was frozen at -80 °C until further processing. RNA was isolated from the frozen dishes by adding RLT lysis buffer (Qiagen, Valencia, CA) to each well and then processed using RNeasy Mini columns (Qiagen) following the manufacturer’s recommended procedure. The quantity and purity of the extracted RNA was evaluated using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and its integrity measured using an Agilent Bioanalyzer. For microarray hybridizations, 1 µg of total RNA was amplified and labeled with a fluorescent dye (either Cy3 or Cy5) using the Low RNA Input Linear Amplification Labeling kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. The amount and quality of the fluorescently labeled cRNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer. Equal amounts of Cy3- or Cy5-labeled cRNA were hybridized to the Agilent Human Whole Genome 44K Oligo Microarray for 17 h, prior to washing and scanning. Data were extracted from scanned images using Agilent's Feature Extraction Software. Gene
expression data were loaded into the Rosetta Resolver® Gene Expression Analysis System. Fluorophore reversal hybridization data were combined using an error-weighted average for each treated sample. PR regulated probesets were identified as those with a p-value < 0.001 and an absolute fold change > 1.3.

5.6.2 H4IIE Cells

Microarray analysis was conducted on 3 biological replicate cultures of H4IIE cells. For each biological replicate, H4IIE cells were seeded on Day 1 at 3.5 x 10^5 cells/well into two wells of a 6-well plate (per treatment) in MEM containing 8% FBS and the appropriate supplements. On Day 2, cells were infected at a multiplicity of infection (MOI) of 80 (for Lx23) or 140 (for LxAA) in phenol red-free MEM containing 8% CS-FBS and the appropriate supplements. After the infection had proceeded for 2.5 h on a rocker at room temperature, the virus/media mixture was removed and the cells were returned to the 37°C incubator overnight in fresh phenol red-free MEM containing 8% CS-FBS and the appropriate supplements. On Day 3, cells were treated overnight with vehicle or 100 nM dexamethasone for 20 h. On Day 4, cells were harvested and duplicate samples (i.e. two wells per treatment) were pooled, and total RNA was isolated as described below. RNA samples were submitted to the Duke University Microarray Core Facility for microarray analysis, as detailed on the following website: <http://www.genome.duke.edu/cores/microarray/services/affymetrix/>. Briefly, the quality of the RNA was assessed using an Agilent Bioanalyzer 2100. Target generation and labeling was performed using the GeneChip 3’ IVT Express kit (Affymetrix Inc.,
Hybridization to Rat Genome 230 2.0 chips was performed per manufacturer’s instructions.

### 5.7 RNA Isolation and Quantitative PCR

Most cells (i.e. T47D variants, BT483) were seeded in 6-well plates in phenol red-free media containing 8% CS-FBS and the appropriate supplements for 48 h. Next, cells were serum-starved for 24 h as described above and treated with the appropriate ligand. For studies with H4IIE cells, the cells were seeded, infected and treated as detailed in the previous section on microarray analysis. After the indicated time period, cells were harvested and total RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA). One µg of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The Bio-Rad iCycler Realtime PCR System was used to amplify and quantitate levels of target gene cDNA. qPCR reactions were performed with 1 µL cDNA, 10 µM specific primers, and iQ SYBRGreen supermix (Bio-Rad). Data are normalized to the 36B4 housekeeping gene (human) or cyclophilin (rat) and presented as fold induction over vehicle. Data are the mean ± SEM for triplicate amplification reactions from one representative experiment. Unless otherwise indicated, each experiment was repeated at least three independent times with very similar results. Human qPCR primer sequences are listed in Table 5.1, and rat qPCR primer sequences are listed in Table 5.2.

**Table 5.1: Human qPCR primer sequences**

<table>
<thead>
<tr>
<th>Human qPCR</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>36B4</td>
<td>Forward: 5’-GGACATGTTGCTGGCCAATAA -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GGGCCCGAGACCAGTGTG -3’</td>
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</table>
Table 5.2: Rat qPCR primer sequences

<table>
<thead>
<tr>
<th>Rat qPCR</th>
<th>Primer Sequence</th>
</tr>
</thead>
</table>
| Cyclophilin | Forward: 5’- AAGGTGAAGAAGGCATGAGCA -3’  
Reverse: 5’- GCCACGGACACGATGAAGTAC -3’ |
| PEPCK    | Forward: 5’- GAATCGCCAGTGAATCCCG -3’  
Reverse: 5’- CATTGTGGCTGTGTGGTC -3’ |
| SGK1     | Forward: 5’- GGTCGCAATGGATGGCTGAC -3’  
Reverse: 5’- TCAGCGAAGAAGGTGAAGTAC -3’ |
| MKP1     | Forward: 5’- AGCTGCTGCAATGGATGGCTGAC -3’  
Reverse: 5’- AAGGTGAAGAAGGCATGAGCA -3’ |

5.8 siRNA Studies

For experiments involving transient transfection of small interfering RNA (siRNA), validated Stealth siRNA or siRNA control were obtained from Invitrogen (see
Table 5.3 for siRNA sequences). Cells were plated in phenol red-free DMEM containing 8% CS-FBS, 0.1 mM NEAA and 1 mM NaPyr in the presence of 40 nM siRNA or siRNA control using DharmaFECT-1 (Dharmacon, Lafayette, CO) as the transfection agent according to the manufacturer's recommendations. After 48 h of knockdown, cells were serum-starved in phenol red-free DMEM containing 0.1% CS-FBS, 0.1 mM NEAA and 1 mM NaPyr for 24 h, then treated with the appropriate ligand and harvested for qPCR analysis as detailed above.

**Table 5.3: siRNA sequences**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siLuciferase</td>
<td>Invitrogen Stealth RNAi proprietary sequence; Catalog #12935-146</td>
</tr>
<tr>
<td>KLF15 #2</td>
<td>5'-GCAUUCUGCUCUGCCCGAGUUCCU-3'</td>
</tr>
<tr>
<td>KLF15 #3</td>
<td>5'-AGGAGAACAUGGAGCCUAGGAGUCAA-3'</td>
</tr>
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</table>

**5.9 Virus Production and Infections**

Adenoviruses expressing β-gal and hPR-B were generated using the ViraPower Adenoviral Expression System (Invitrogen) and were amplified and purified by CsCl₂ centrifugation. For adenovirus infection, hMECs were seeded in 6-well plates in normal media for 48 h, and then serum-starved with 0.001% serum media without Epidermal Growth Factor (EGF) for 36 h. Cells were infected at a multiplicity of infection (MOI) of 150 in the absence or presence of hormone added 90 min post-infection, and RNA was isolated 16 h after treatment and subjected to qPCR analysis as described above.
5.10 Proliferation Assays

For BrdU incorporation assays, T47D:A18 cells were seeded on Day 1 at 5 x 10³ cells/well in 96-well plates using phenol red-free DMEM containing 8% CS-FBS, 0.1 mM NEAA and 1 mM NaPyr. On Day 3, cells were serum-starved for 24 h as previously described. On Day 4, the cells were treated overnight with ligand in the indicated media conditions (phenol red-free DMEM containing 0.1% CS-FBS, 10% CS-FBS or 10% FBS). On Day 5 (18 h post-treatment), the BrdU labeling reagent was added for 4 h. Cells were then assayed according to manufacturer’s protocol (Cell Proliferation ELISA BrdU Kit, Roche Applied Science, Indianapolis, IN). Data is presented as the mean ± SEM for triplicate wells in one representative experiment.

5.11 Western Blotting

T47D:A18 cells were seeded in 10-cm plates in phenol red-free DMEM containing 8% CS-FBS, 0.1 mM NEAA and 1 mM NaPyr for 48 h, after which the cells were serum-starved for 24 h as described above. For studies with H4IIE cells, the cells were seeded, infected and treated as detailed in the previous section on microarray analysis. Following treatment with the appropriate compound for the indicated time periods, cells were harvested in ice-cold PBS and lysed in RIPA Buffer [50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 50 mM NaF, 2 mM Na₃VO₄, and 1X protease inhibitor mixture (EMD Chemicals, Inc, San Diego, CA)] while rotating at 4°C for 30 min. 20 µg of whole-cell extract was
resolved by SDS-PAGE, transferred to a PVDF membrane (Bio-Rad) and probed with the appropriate antibodies. Results shown are representative blots.

5.12 Chromatin Immunoprecipitation (ChIP)

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. Cells were grown to 90% confluence in phenol red-free DMEM supplemented with 8% CS-FBS, 0.1 mM NEAA and 1 mM NaPyr for 48 h, after which the cells were serum-starved for 24 h as described above. Following treatment with the appropriate ligand for the indicated time periods, cells were subjected to ChIP analysis as previously described (39), with the following modifications. Immunoprecipitation was performed overnight at 4°C with 10 µg PR-specific antibody (1294, D. Edwards, Baylor College of Medicine), 10 µg E2F1-specific antibody (KH95, Santa Cruz Biotechnology) or 10 µg mouse IgG control (Santa Cruz Biotechnology). After immunoprecipitation, 70 µl Protein A/G-PLUS-Agarose beads (Santa Cruz) [50% slurry in 10 mM Tris-HCl pH=8.0, 1 mM EDTA] was added and allowed to incubate for 3 h at 4°C. qPCR analysis was performed as described above, and data is normalized to the input for the immunoprecipitation. ChIP primer sequences are listed in Table 5.4.

Table 5.4: ChIP primer sequences

<table>
<thead>
<tr>
<th>ChIP</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stromelysin</td>
<td>Forward: 5’- TCTATCCCAAGCTGAAGAACTGGCCAGTCCCTGC -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CAAGTAGCTGGGACCACAGACGTGCGCCACCATG -3’</td>
</tr>
<tr>
<td>FKBP51</td>
<td>Forward: 5’- AACACCCCTGTCTGAATGTGGGCTG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- GAGCTAATGTCTTTTAGGCTGGAGCA -3’</td>
</tr>
<tr>
<td>E2F1 promoter (E2F binding sites)</td>
<td>Forward: 5’- AGGAACCGCCGCCGTTGTTCCCGT -3’</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>E2F1 Proximal PRE #1</td>
<td>Forward: 5’- GCTGGGAGTACTTATGTGGGA -3’</td>
</tr>
<tr>
<td>E2F1 Distal PRE #3</td>
<td>Forward: 5’- CATTGCCACAGATCCCCAAA -3’</td>
</tr>
<tr>
<td>E2F1 Distal PRE #4</td>
<td>Forward: 5’- CCTGCAGGGTGCTAAGGATA -3’</td>
</tr>
<tr>
<td>E2F1 Distal PRE #5</td>
<td>Forward: 5’- AAATCAGACCACAGCAGCAG -3’</td>
</tr>
<tr>
<td>E2F1 Distal PRE #6</td>
<td>Forward: 5’- GGCTGTTTGGGACTCCTCAT -3’</td>
</tr>
</tbody>
</table>
6 Appendix: Dissecting the role of AF-1 versus AF-2 in glucocorticoid receptor-mediated gene regulation

6.1 Introduction

Glucocorticoids (GCs) are key regulators of many physiological processes, including glucose homeostasis, development and the immune response. Synthetic GCs such as dexamethasone have many beneficial therapeutic uses, especially in the suppression of inflammatory responses in disease pathologies such as asthma, rheumatoid arthritis, prevention of graft rejection and autoimmune diseases (176). However, GCs can have many harmful side effects, including osteoporosis, muscle wasting, hypertension and diabetes; furthermore, prolonged exposure to GCs can eventually result in GC sensitivity and resistance (162).

The effects of GCs are mediated by the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-activated transcription factors [reviewed in (219)]. In the absence of ligand, GR resides in the cytoplasm of target cells, where it is maintained in an inactive state through association with heat shock proteins and immunophilins. Upon ligand binding, GR undergoes a conformational change that leads to its dissociation from the chaperone complex, an event that exposes nuclear localization signals and facilitates receptor translocation into the nucleus. Next, receptor homodimers interact with specific glucocorticoid-responsive elements (GREs) within target gene promoters. The DNA-bound receptor subsequently nucleates the assembly of large cofactor containing protein complexes that can either positively or negatively impact
gene transcription. Alternatively, GR can impact downstream gene expression through cross talk with other transcription factors, including nuclear factor-kappa B (NF-κB), signal transduction and activator of transcription (STAT), Sma and Mad-related protein (Smad) and activator protein-1 (AP-1).

Human GR contains two transcriptional regulatory domains: activation function (AF) 1 and 2. The ligand-independent AF-1 domain is located in the N-terminal region of GR, whereas the highly conserved, ligand-dependent AF-2 domain can be found in the C-terminal ligand-binding domain (LBD) (50). While much progress has been made in elucidating the mechanisms by which AF-1 and AF-2 differentially regulate the expression of GR target genes, the relative contributions of AF-1 vs. AF-2 to downstream GR signaling as a whole remain poorly understood. This is largely due to the fact that mutations of AF-2 affect other aspects of GR function besides coactivator binding, including conformation, dimerization, and AF-1 function.

Through combinatorial phage display, we have identified a 21 amino acid peptide named Lx23 that interacts strongly with dexamethasone-bound GR (data courtesy of Dr. E. Kimbrel, Duke University, Durham, NC). Lx23 contains a canonical ‘LXXLL’ motif which allows it to interact with the AF-2 coactivator binding pocket of agonist-bound GR and consequently block any downstream signaling that is modulated by AF-2 (Figure 6.1). Transcriptional interference assays have shown that Lx23 acts as a potent suppressor of both exogenous and endogenous GR activity; however, Lx23 does not affect
expression of endogenous GR at the mRNA or protein levels (personal communication with Dr. M. Tochacek, Duke University, Durham, NC).

Figure 6.1: GR structure

Schematic illustration of GR modular structure, highlighting the region where the Lx23 can bind and thereby block GR signaling. N (amino-terminus), AF (activation function), DBD (DNA-binding domain), NLS (nuclear localization signal), H (hinge region), LBD (ligand-binding domain), C (carboxy-terminus). Lx23 peptide sequence identified by Dr. E. Kimbrel.

6.2 **Hypothesis & Objectives**

We hypothesize that expression of the Lx23 peptide in H4IIE cells will inhibit the ability of GR to regulate target gene transcription through AF-2 signaling, while leaving the AF-1 functions of GR intact. Thus, by comparison to cells without Lx23 expression, we will be able to identify novel GR target genes that are regulated by AF-1, AF-2 or both domains.

6.3 **Preliminary Data**

To determine whether Lx23 can be utilized as a tool to differentiate between AF-1 versus AF-2 actions in GR-mediated gene regulation, we carried out studies to assess the effects of the synthetic GC dexamethasone on induction of several GR target genes in the presence or absence of Lx23. We chose to use the H4IIE rat hepatoma cell line as our
model system, since it contains intact gluconeogenic and inflammatory pathways that are responsive to GCs. Prior to treatment with dexamethasone, H4IIE cells were infected with adenoviruses expressing either Lx23 or LxAA, a control peptide that cannot bind to AF-2 of GR because the leucine residues in its ‘LxxLL’ motif were mutated to alanines. LxAA should not impact the AF-2 functions of GR, and thus it serves as an additional control that can be utilized to identify any non-specific effects that are due to infection, and not to inhibition of AF-2 activities.

As expected, our qPCR analysis of H4IIE cells treated with dexamethasone in the presence or absence of Lx23 revealed two distinct classes of GR target genes. Class I consists of GR target genes that are AF-2 regulated, or inhibited by Lx23 in a dose-dependent manner, such as phosphoenolpyruvate carboxykinase (PEPCK) and serum- and glucocorticoid-inducible kinase 1 (SGK1) (Figure 6.2). Conversely, Class II contains genes whose regulation by GR is not AF-2 dependent, or not affected by Lx23, such as MAPK phosphatase 1 (MKP1) (Figure 6.3).
Figure 6.2: Class I GR target genes: regulated by AF-2, inhibited by Lx23

H4IIE rat hepatoma cells were mock-infected (no virus) or infected with increasing amounts of adenoviruses expressing Lx23 or the control LxAA peptide. Next, cells were treated with vehicle (veh) or 100 nM dexamethasone (Dex) for 20 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. PEPCK or SGK1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene cyclophilin. Results are expressed as fold induction over mock-infected, vehicle-treated cells ± SEM (n=3). MOI (multiplicity of infection).
H4IIE rat hepatoma cells were mock-infected (no virus) or infected with increasing amounts of adenoviruses expressing Lx23 or the control LxAA peptide. Next, cells were treated with vehicle (veh) or 100 nM dexamethasone (Dex) for 20 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. MKP1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene cyclophilin. Results are expressed as fold induction over mock-infected, vehicle-treated cells ± SEM (n=3). MOI (multiplicity of infection).

### 6.4 Experimental Design

Having established the differential effects of Lx23 on a small panel of GR target genes, we set out to obtain a more global view of Lx23 actions on GR gene regulation. Towards this end, we carried out a microarray experiment with the following design (Figure 6.4). Three replicates of H4IIE cells were subjected to mock infection conditions, or infection with adenoviruses expressing either the LxAA control peptide or Lx23, and then treated with vehicle or 100 nM dexamethasone for 20 h. Prior to exploring Lx23 effects on GR gene regulation, we utilized Western blot analysis to confirm equal expression of the two peptides, and to verify that Lx23 does not affect total GR protein levels (Figure 6.5). Next, qPCR studies were performed to confirm that Lx23 had similar
effects on the three target genes that were previously examined (Figure 6.6). Finally, the RNA samples were subjected to microarray analysis.

**Figure 6.4: Microarray experimental design**

Flow chart depicting the design of our H4IIE microarray experiment.

**Figure 6.5: Lx23 does not affect endogenous GR protein expression in H4IIE cells**

H4IIE rat hepatoma cells were mock-infected (no virus) or infected with adenoviruses expressing Lx23 or the control LxAA peptide, fused to Gal4DBD. Next, cells were treated with vehicle (veh) or 100 nM dexamethasone (Dex) for 20 h. After treatment, cells were harvested and 20 µg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for total GR, Gal4DBD or GAPDH as a loading control. A representative blot is shown (n=3).
Figure 6.6: Effects of Lx23 on GR target gene expression

H4IIE rat hepatoma cells were mock-infected (no virus) or infected with adenoviruses expressing Lx23 or the control LxAA peptide. Next, cells were treated with vehicle (veh) or 100 nM dexamethasone (Dex) for 20 h. After treatment, cells were lysed and RNA was isolated and reverse transcribed. PEPCK, SGK1 or MKP1 mRNA levels were quantified using qPCR and normalized to the housekeeping gene cyclophilin. Results are expressed as fold induction over mock-infected, vehicle-treated cells ± SEM (n=3).

6.5 Expected Outcomes

In future bioinformatic analyses of the microarray data, we will compare the profile of genes that are expressed after vehicle or dexamethasone treatment in the presence and absence of the Lx23 peptide. We expect to identify novel genes whose regulation by GR is controlled by AF-1, AF-2, or both domains. After validation of these GR target genes by qPCR analysis, GO-term analysis will be used to determine if AF-1
or AF-2 regulated gene clusters correlate with specific downstream biological pathways that are modulated by GR. If such correlations are identified, we could potentially explore whether specific pharmaceutical manipulations of distinct GR regulatory pathways could selectively suppress certain processes, especially inflammatory responses, but simultaneously avoid or limit any unwanted side effects such as bone loss or muscle wasting.
References


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estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288:321-33.


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Fellowships: